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LINKAGE OF CHROMOSOMES CORRELATED WITH REDUCTION IN NUMBERS AMONG THE SPECIES OF A GENUS, ALSO WITHIN A SPECIES OF THE LOCUSTIDÆ.

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In the following paper it is my purpose to give the result of my study of the chromosome numbers within three species of one genus of the Jamaican *Locustidæ*.

The material used was collected in Jamaica during the summer of 1912 by Professor W. R. B. Robertson, of Kansas University. The testes of all but one individual, No. 416, were removed while in the field and fixed in either Bouin's or Flemming's fluid. The slides were made in the fall of 1912. The clearest and best division figures as well as those in which the chromosomes were most crowded together and unsatisfactory were made from Flemming-fixed specimens. The sections were cut at about twelve micra and iron-hematoxylin proved most satisfactory for staining. All drawings have been made at the level of the base of the microscope with the aid of a camera lucida. A 2 mm. Spencer oil immersion lens and No. 18 Zeiss compensating ocular were used. All the figures were magnified to 3,900 diameters and have been reduced 1/5, giving a final magnification of 3,120 diameters.

The specimens studied were identified and classified by Mr. A. N. Caudell of the United States National Museum, Washington, D. C., as follows:

588 and 589, adult males of *Jamaicana flava*, N. Sp., Caudell.

430, a nymph and 560, an adult male of *Jamaicana unicolor*, Brunner.

416, 585, 586, 587, four adult males of *Jamaicana subguttata* Walker.

438, a nymph and 503, a small male of *Jamaicana subguttata*.

In determining the number of chromosomes in the various individuals, I have, in all cases, made drawings from not only different cysts, but from different follicles as well. In a few instances where the figures are particularly clear and distinct, I have taken more than one cell from the same cyst (Nos. 30, 31, 33) but where this was done I made additional drawings from other parts of the testes. There can be no doubt as to the character or appearance of these figures.

Observations.

Jamaicana flava n. sp. Caudell.

All chromosomes are of the rod type. One individual shows two pairs of peculiarly associated chromosomes in the spermatogonial division figures.

In my comparison of species, the description of *J. flava* should logically come first since the chromosomes here are all of the rod shaped, simple type, and are not sufficiently associated to form multiples. They also vary the least in their behavior from what we might consider the original or primitive condition of the species of this genus. To distinguish individuals of the same species, I shall refer to each by number. Nos. 589 and 588 belong to *J. flava*.

In individual 589, I found the number of chromosomes to be thirty-five. They are all rod-shaped, varying in size from the large unpaired accessory chromosome which I have numbered 18, through a graded series of pairs indicated by number, according to their size from 17 to 1.

In a polar view of a spermatogonial metaphase figure, the seventeen or eighteen largest chromosomes are found, as a rule, on the periphery in no fixed position or order, with the smallest pairs clustered about in the center. This is quite well shown in Figs. 3 and 4 while Figs. 1 and 2 show some of the larger chromosomes in the center of the figure where they have probably displaced the smaller ones through some accident in previous mitoses. There seems to be nothing unusual or irregular in the number or relation of chromosomes here either in spermatogonial or spermatocyte divisions.

In the first spermatocyte stages the chromosome complex consists of seventeen undivided autosomes plus the accessory or sex-chromosome (Figs. 10 and 11). As division proceeds, the unpaired sex-chromosome goes entire to one pole accompanied or followed by 17 autosomes. The mates of these seventeen autosomes pass to the opposite pole, thus making the number at the two poles eighteen and seventeen respectively. Before this division takes place, the paired autosomes show the same gradation in size as was noted in spermatogonial figures (Fig. 11). There are several extremely large chromosomes in this individual but the change in the series is so gradual that it is difficult to say where the dividing line falls. However, I am reasonably sure I can pick out at least three pairs in the spermatogonial or three undivided chromosomes in the first spermatocyte cells, that are larger than the others.

In 588, the second individual of *J. flava* studied, a slight but distinct difference can be noted in the spermatogonial figures. The number of chromosomes is the same, thirty-five, and all are of the rod type varying in size as was noted in No. 589. However, in pairing off the chromosomes, I find there are in each cell, two pairs of closely related ones. Each member of the pair No. 16 is always found in close association with a member of the pair No. 14 (*A*, Figs. 5, 6, 7, 8). The members of the other pairs are distributed throughout the figure very much the same as in corresponding figures of No. 589. The largest chromosomes always appear on the periphery and the smallest ones in the center. These two sets of parallel rods, as may be noted, in the figures, have no regular position in respect to each other or to other chromosomes in the cell. Sometimes they are near each other and as often they are to be found on opposite sides of the figure. However, they are never far from the accessory chromosome.

Jamaicana subguttata Walker.

Five individuals out of six belonging to *J. subguttata* show only the rod type of chromosomes. In the sixth individual, a distinct variation is found in the appearance of a V-chromosome.

Of the ten individuals I studied, numbers 416, 585, 586, 587, 438 and 503 belong to *Jamaicana subguttata*. The sections of

No. 416 broke up badly so that it was difficult to find a perfect division figure. However, a sufficient number were found to determine the number and character of the chromosomes (Figs. 13–14).

I found no distinctive feature in the number, arrangement, or behavior of the chromosomes in the first five individuals of this species (Figs. 13 to 29). There are thirty-five chromosomes in each and their appearance is very similar to that noted in individuals, No. 588 and 589. The accessory chromosome is very prominent and in the majority of spermatogonial metaphase figures it is found with seventeen of the largest autosomes on the periphery, the remaining seventeen small ones being in the center of the figure (Figs. 13, 15, 25–29).

A most interesting feature was found however in the individual No. 503. Instead of the thirty-five autosomes of the simple rod type that were common to the other members of the genus as well as species, I found here thirty-three rods and a large V-multiple whose arms are of unequal length. When I attempted to arrange the autosomes in pairs, I found two of the large ones, numbers 16 and 14, without mates among the rod type but corresponding in size to the arms of the V (Figs. 30 to 33). In one cyst I found several perfect cells in spermatogonial metaphase showing this size relation between the rods and the arms of the V (Figs. 30–31–33).

After satisfying myself that this multiple chromosome appears in all spermatogonial metaphase figures, I examined cells in other stages of growth. In the various phases of the first and second spermatocyte cells I found the V still present. In the first spermatocyte figures the rod mates were often still attached to the arms of the V but the break could always be distinguished more or less distinctly (Figs. 37 through 50). In first spermatocyte metaphase (Fig. 42), I found the V still attached to or united with its mates as were several of the rod chromosomes also. In this stage I found fifteen rods without the two in the V and the accessory, making in all eighteen chromosomes in the first spermatocyte.

In the second spermatocyte division the accessory chromosome divides longitudinally so that each daughter cell receives half.

The same kind of division evidently takes place in the V-multiple here. In Fig. 52, we can see the one V going over to one pole a little after the other chromosomes have passed. What appears to be the other half of the V has already reached the opposite pole. I did not find another cell showing this conclusively but the division of the other parts of the chromatin matter would seem to indicate this interpretation. The V at the pole is again the size of the multiple in the spermatogonial stage.

Jamaicana unicolor Brunner.

The two individuals of *J. unicolor* differ. One is of the simple rod type, while the other shows the two V-chromosomes.

The spermatogonial metaphase figures of individual 430.1 show thirty-five chromosomes of the rod type (Figs. 54–57). There is nothing in their appearance or behavior to distinguish them from individual 589 of *J. flava* or from individuals 416, 585, 586, 587 and 438 of *J. subguttata* and the same holds true in later stages of growth.

In No. 560.1, the second individual of this species, I found two multiple chromosomes (Figs. 58–67). In addition to the thirty rod autosomes, the accessory and two large V's are to be seen on the periphery of the spermatogonial metaphase figures. The chromatin material in this series of slides is so badly massed that perfect cells are difficult to find. For this reason I can not be certain of the size relations among all the chromosomes but I have gained the essential facts for my purpose. The largest chromosomes are quite clear and distinct so that I can number the mates of the five largest pairs besides the accessory. Figs. 58–60 show the number and arrangement of the chromosomes. Many spermatogonial cells can be found in which the two V's and the accessory are readily distinguishable although the other chromosomes may be too badly massed for further study (Figs. 61–64).

In the first spermatocyte, the multiples are found attached to each other end to end, thus forming an elongated ring. A slight constriction appears where the ends of the V's meet (Figs. 65–66).

The cells in the earlier growth stages were small and the chromatin so massed together that I gained nothing from them.

Discussion.

McClung and others who have worked on the chromosomes of the *Orthoptera* believe there is a fixed or definite number for each group of related insects, and presumably for all kinds of life. Other writers disagree. Miss Browne ('13) has summarized the work done and results obtained by many investigators on varied kinds of life so thoroughly that I shall not go into this in detail. I shall, however, review the methods as set forth by her whereby the changes in the chromosome numbers have been accounted for by the various authors.

One method is by the fusion or separation of particular chromosomes. Miss Browne in her work on *Notonecta* ('13) and Wilson ('11) on *Nezara* use this explanation. A change in number by a process of fusion was used by McClung ('05) and Robertson ('15) in the appearance of a multiple chromosome, the former in *Hesperotettix* and *Mermiria*, the latter in *Chorthippus* (*Stenobothrus*) *curtipennis*. A change by a process of splitting has been advocated by several observers, Payne ('09) and Wilson ('11) among them. These account for slight or gradual changes but wide variations are accounted for by Wilson by a new segregation of the nuclear material causing a change in number and size relations of the chromosomes, but not in their essential quality. Another method whereby a change might take place is by an abnormality occurring in mitosis. Wilson ('09*a*) has described an unequal distribution of the chromosomes to the daughter cells in *Metapodius*. An arrest of cell division after a division of the chromosomes has taken place was found by Boveri ('05) in sea-urchin eggs.

My material resembles McClung's ('05) in that the change in number is accounted for by the fusion of chromosomes, these giving rise to a multiple (V). However, the composition and behavior of this multiple differs from the one described by him in so far as its relation to the sex chromosome is concerned.

These multiple or V-chromosomes resemble very much more those being described by Robertson ('15) in *Chorthippus* (*Steno*) *bothrus*). Robertson has found that *Chorthippus* (*Stenobothrus-curtipennis* has seventeen chromosomes and of these, six are V's (three pairs of V's). Counting each limb of the V as a chromo-

some, he believes there results twenty-three, the number normal for *Truxalinæ* a subfamily of the *Acrididæ*. It was at his suggestion that I undertook to determine if *Jamaicana* which I found to have these V-shaped chromosomes showed the same phenomena.

In my material the multiple is likewise apparently formed by the union of two of the autosomes. As has been done by others, I numbered and paired the chromosomes according to size, calling the unpaired accessory which is the largest chromosome in the complex, No. 18, and grading the others down from that. I at once found two autosomes of unequal length, No. 16 and 14, without mates as well as the very noticeable V. Since the arms of the V are unequal, and correspond in size as well as length to the unmated rods, I believe No. 16 is the mate of the long arm of the V, while No. 14 mates with the short arm of the multiple (Figs. 30–36). The sex chromosome is very prominent and is often seen in close proximity to the multiple throughout the transitional stages, but it never unites with the components of the multiple as McClung found in *Hesperotettix* and *Mermiria*.

In the spermatogonial metaphase cells the V first appears. It is always on the periphery of the ring with the apex pointing inward, while its rod mates may be anywhere on the periphery, sometimes near it, sometimes opposite it.

From the spermatogonia through the spermatocytes this multiple can be traced. It divides longitudinally in spermatogonia. In the first spermatocyte the tetrads divide transversely separating the V from its rod mates. Half of the second spermatocyte cells receive the V and half receive the rod mates of the V (Figs. 37–47). In the second spermatocyte we have every reason to believe that the V divides longitudinally, each of the two spermatids resulting thus receiving a V while from the other second spermatocyte two spermatids result which will contain the rod mates of the V.

This is what takes place in the one V-type of *J. subguttata*. The associated rods in No. 588 *J. flava* (Figs. 5–8) are easily distinguishable in the polar view of the spermatogonial metaphase and their size 16 and 14 in the series corresponds to that of the arms of the V in No. 503. Their behavior can not be traced as

readily as the V, however, since their union is evidently not permanent and the rods separate in the later growth stages. The fact that they are associated in the early stage forming two sets of related rods on the periphery strengthens my belief in the manner of the V-formation in the other forms. In the place of these two sets of rods, or of the one V and one set of rods in No. 503, I found two V's in No. 560 a representative of *J. unicolor*. Here we find the same thing taking place as in the one V type. In the place of a long V formed by the short V and the rod mates in the spermatocyte figures, we here find a ring formed by an end-to-end union of the arms of the V's (Figs. 65–67).

It will be seen that throughout the three species of this genus of Jamaican *Locustidæ* the number of chromosomes, 35 remains constant, although their behavior varies not only within the genus but even within the species. In seven of the ten individuals studied representing three species, there are 35 chromosomes of the simple rod type. As a whole, the division figures are clear and distinct and the growth stages can be followed with moderate certainty. In the first spermatocyte, the accessory passes undivided to the one pole making the number in the two daughter cells 18 and 17 respectively. When these divide in the second spermatocyte, division takes place longitudinally and the spermatids number 18, 18, 17 and 17 respectively, the last two being without the accessory chromosome. These then will produce males when fused with eggs, and the oosperm will contain thirty-four autosomes plus the one sex chromosome brought in by the egg. Those spermatids with eighteen, will produce females, since here there will be two sex chromosomes, one from the sperm and one from the egg in addition to the thirty-four autosomes, making the number 36 in the females.

I find, however, an exception from this general rule of 35 separate rod type chromosomes in each species studied. *J. flava* shows in one individual the presence of two pairs of associated rods in all spermatogonial figures. The rods that make up each pair or set are unequal and hence can not be mates. In numbering the members of the complex these rank as 16 and 14 in the graded series, 18 being the unpaired accessory and the largest

in the group (Figs. 5–8). I find by numbering the members of the pairs as separate individuals, we have thirty-five chromosomes here as in the other seven forms. In the later stages of growth, nothing appears to distinguish this form from its fellow No. 589 so we must conclude the rods of the related pairs are not held permanently together but are lost after the spermatogonial division among the other rods of the complex.

In *J. subguttata* a multiple chromosome appears resembling a V which I believe has been formed by the union of two of the autosomes. These, by continued association, have finally united end to end and we find them forming a V. The members or arms of the V are unequal and, as was noted in connection with the associated rods, can not be mates, since mates are identical. Hence the mates to the components of the multiple must be rods. I numbered and paired the chromosomes in the spermatogonia according to size and found two rods 16 and 14 without rod mates. These correspond with the arms of the V in size and appearance, and as many cells show the same relation, it seems to me they may be so taken. Figs. 30–36.

The inequality of the parts of the multiple is especially well shown in the first spermatocyte figures. The long arm of the V is linked up with a long rod of approximately the same size, while a somewhat shorter or smaller rod is united with the smaller arm of the V. A slight constriction is to be seen where the rods join the arms of the multiple.

In determining the count for this species, those individuals having the simple rod type chromosomes, have of course thirty-five in their complex. In No. 503, the odd one in this group, there are thirty-three chromosomes of the rod type plus the multiple which I believe is composed of two of these autosome rods united. This then makes the number for the entire species thirty-five, the same as was found for *J. flava*.

The exception in *J. unicolor* contains two multiples or V's similar to the one found in *J. subguttata*. They are apparently formed in the same way and probably of the same two chromosomes, Nos. 16 and 14, as the difference in the lengths of the arms of the V's corresponds with that in No. 503. In the first maturation division, the V's which compose the elongated ring,

break apart at the constriction and each cell receives one V. This splits longitudinally in the next division so that each spermatid carries one V multiple.

J. unicolor contains thirty-one rods and the two multiples. If we accept what has apparently taken place in the previous cases, we can now say the V multiples are composed of four rods which will make the number for the individual thirty-five.

It seems evident then, that the ten individuals, representatives of three distinct species, contain a uniform number of chromosomes regardless of the fact that their behavior differs by the appearance of multiples in two specimens and a transitional form containing associated rods, not yet forming a V, in one individual.

TABULATED RESULTS.

Species.	Accession Number of Specimen Studied.	Number of Free Rod-type Chromosomes.	Number of Groups of Rods Associated by 2's.	Number of V's.	Total Number of Rod Elements (Chromosomes).
J. flava.....	588	31	2	none	35
	589	35	none	none	35
J. subguttata.....	416	35	none	none	35
	438	35	none	none	35
	503	33	none	1	35
	585	35	none	none	35
	586	35	none	none	35
	587	35	none	none	35
J. unicolor.....	430	35	none	none	35
	560	31	none	2	35

Although I made my drawings by the aid of a camera lucida I determined the series of size relations from the slide as well as from the drawing, comparing and judging as accurately as I could the graded pairs. As a result of this study, I believe it is the same 14–16 pair that we find associated in the individuals described, Nos. 588, 503, and 560. Just what significance this may have upon the life processes of these grasshoppers I can not say. As to outer body characters, there is nothing to indicate a variation from those of the simple rod type.

There is still another interesting association in this material that I feel should be noted here. In the cases where a multiple is present, it is more or less closely attended by the extremely large accessory chromosome. In the No. 503 material, this

really occurs more often than my drawings would indicate. Whenever the multiple is seen, whether in an entire cell or only in a section, it is quite rare that the sex chromosome does not accompany it. In the majority of cases, although my drawings do not bear me out in this, spermatogonial figures show these two elements on the same half of the periphery. The No. 560 material shows this particularly well. They are not connected as McClung found in *Mermiria* ('05), and Robertson in *Chorthippus* ('15) but seem to be influenced or attracted by each other so that they are generally found in close proximity.

It seems to me the individuality or genetic continuity of the chromosomes which Wilson ('09) speaks of, is pretty well established here in at least the multiple types. In cysts that show different stages of division, the V may often be distinguished still intact, so that it can be traced from spermatogonia through the second spermatocyte still in the V form. It is quite likely only one half the spermatids of the one-V type receive a V while each spermatid of the two-V type receive one.

I wish to express my thanks and indebtedness to Prof. W. R. B. Robertson for his helpful suggestions and encouragement during the progress of this work.

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September, 1914.

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PLATE I.

Jamaicana flava n. sp. Caudell.

In polar views of spermatogonial metaphase figures, the number of chromosomes in both individuals is thirty-five. Chromosomes are paired and numbered according to size, 18 being the single accessory.

FIGS. 1–4. Polar view of spermatogonial metaphase of individual No. 589.

FIGS. 5–8. Polar view of spermatogonial metaphase of individual No. 588, showing, *A*, the oddly related pairs of chromosomes (14's and 16's) in each figure.

FIG. 9. Lateral view of spermatogonial anaphase. No. 588.

FIG. 10. Polar view, first spermatocyte of individual 589.

FIG. 11. Lateral view of metaphase of first spermatocyte chromosomes numbered according to size. Sex-chromosome going undivided to one pole. Individual 589.

FIG. 12. Prophase of second maturation division or interkinesis.

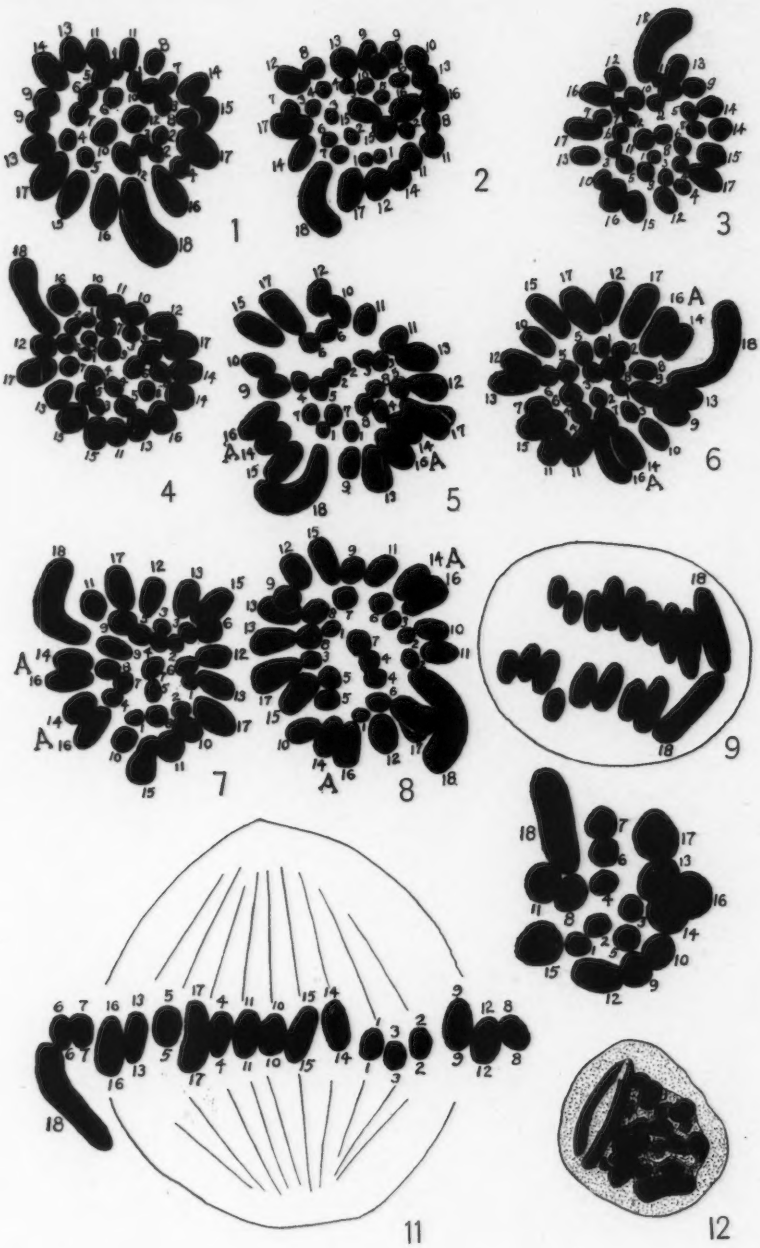
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PLATE II.

Jamaicana subguttata Walker.

Spermatogonial figures from four individuals (Fig. 19 exception). The number in each polar view is thirty-five. Chromosomes are paired and numbered according to size, 18 being the accessory.

FIGS. 13–14. Spermatogonial figures from individual 416. Polar view, metaphase.

FIGS. 15–18. Polar view of spermatogonial metaphase figures from individual No. 585.

FIG. 19. Lateral view of first spermatocyte. The full number of chromosomes is not shown.

FIGS. 20–22. Polar view of spermatogonial metaphase figures from individual No. 586.

FIGS. 23–26. Polar view of spermatogonial metaphase figures from individual No. 587.

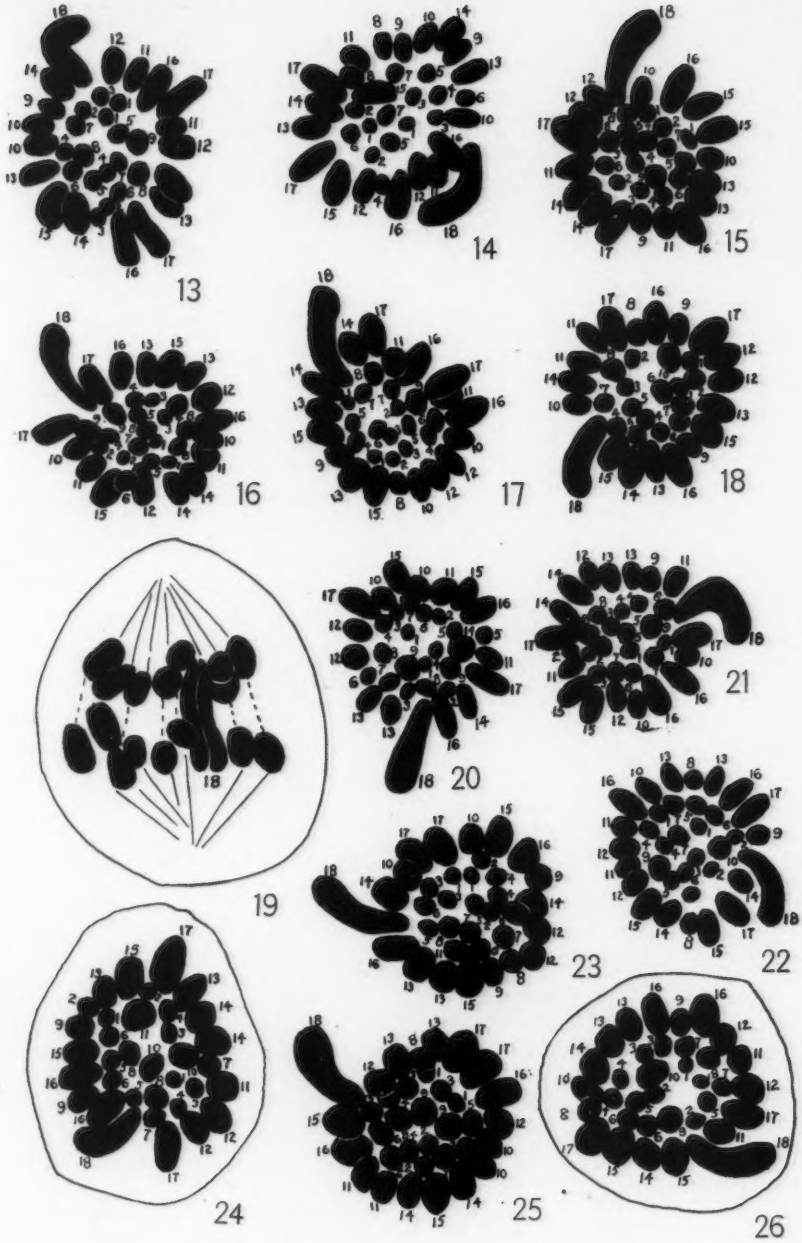
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PLATE III.

Jamaicana subguttata Walker.

Spermatogonial figures from two other individuals of this species. The number of chromosomes in 438, is thirty-five. In 503 there are thirty-three simple chromosomes plus a V-shaped multiple.

FIGS. 27–29. Polar view, metaphase spermatogonial figures of individual No. 438.

FIGS. 30–36. Polar view, metaphase spermatogonial figures of No. 503. The V-multiple chromosome is conspicuous on the periphery.

FIG. 37. First spermatocyte, late prophase showing the long V bi-tetrad of the one-V type.

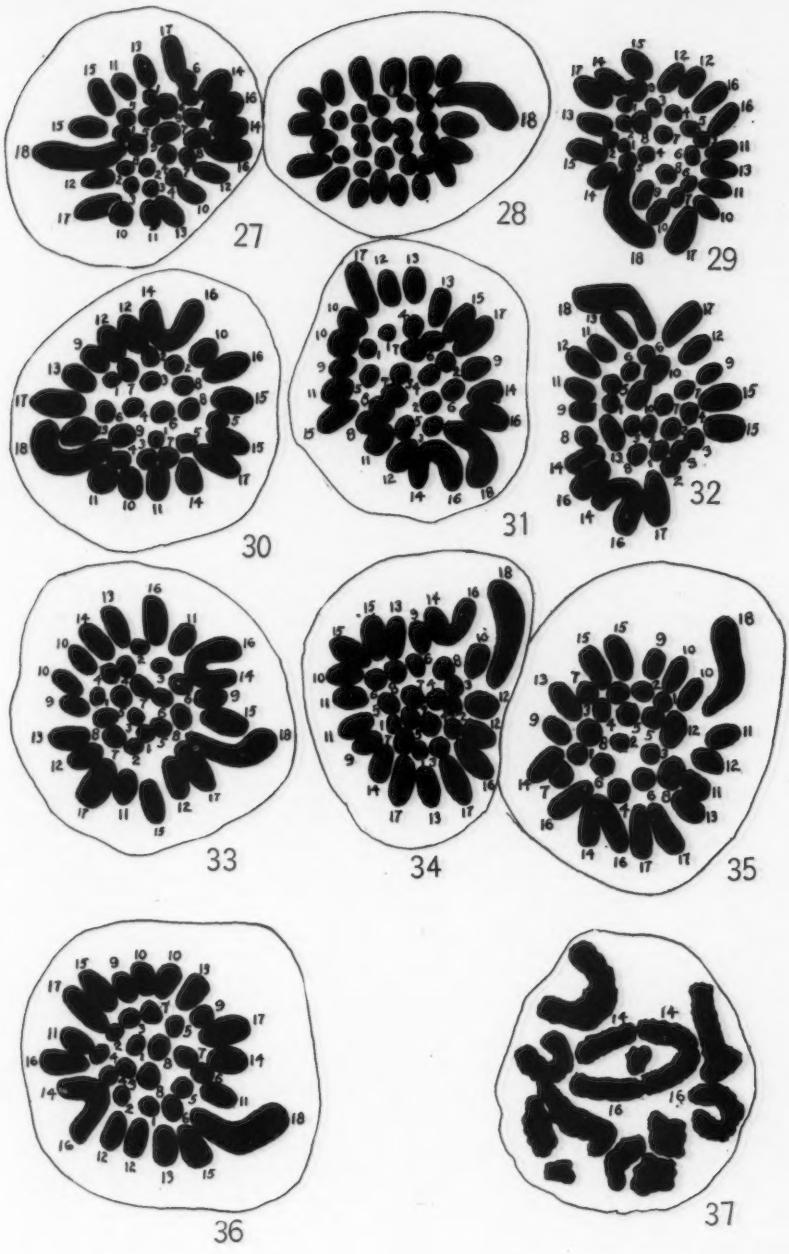
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PLATE IV.

Jamaicana subguttata Walker.

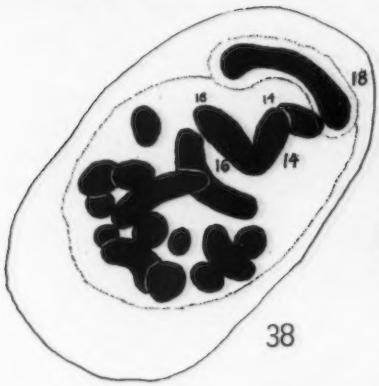
First spermatocyte figures of No. 503.

FIG. 38. Polar view prophase of first spermatocyte showing fifteen simple autosomes, the V-multiple, and the "accessory" chromosome. The latter is enclosed in a receptacle apart from the other chromosomes.

FIGS. 39–41. Lateral view metaphase of first spermatocyte showing the multiple chromosome. The constrictions in the arms of the V show where the rod mates are about to pass to the opposite pole, breaking away from the multiple at the constricted places. The full number is not present but the accessory is prominent in 39 and 40.

FIG. 42. A spermatocyte figure showing the full number, 15, of tetrad chromosomes plus the double tetrad, plus the sex chromosome.

FIG. 43. First spermatocyte showing double tetrad and the accessory chromosome.



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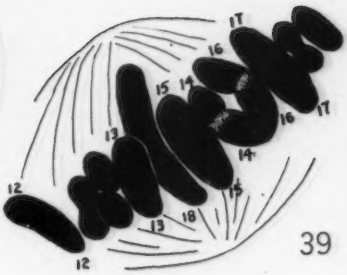

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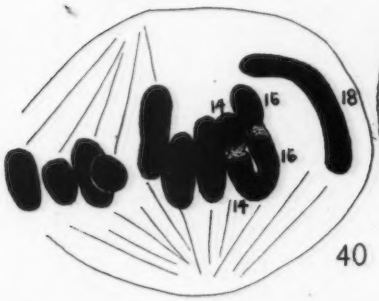

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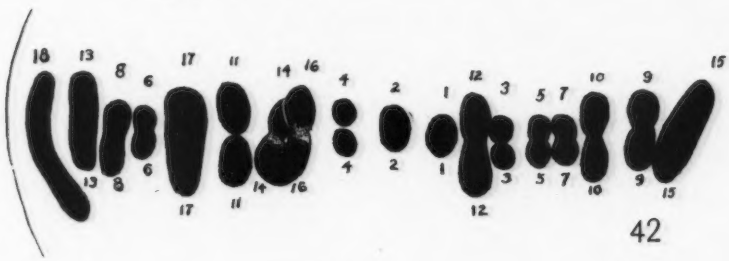

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PLATE V.

Jamaicana subguttata Walker.

Spermatocyte figures of No. 503.

FIGS. 44–46. First spermatocyte anaphase. The multiple chromosome is to be seen in each figure. It has lost its rod mates, which have probably gone to the opposite pole, and is now the size of the spermatogonial V. By comparing it with the multiple before division as seen in Figs. 39–43, what has taken place, is more readily seen.

FIGS. 47. First spermatocyte telophase showing the V at one pole and its rod mates and the sex chromosome at the other. The latter is split longitudinally.

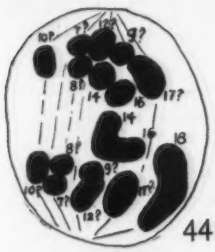
FIG. 48. Telophase of a first spermatocyte division.

FIG. 49. Telophase of first spermatocyte division containing the sex-chromosome and the multiple.

FIGS. 50–51. Telophase of the first spermatocyte or resting period of the second spermatocyte. The sex-chromosome is split preparatory to the second spermatocyte division.

FIG. 52. Second spermatocyte. Sex-chromosome divided. The V-chromosome is here divided and the arms are of the original size seen in the spermatogonial figures.

FIG. 53. Second spermatocyte. Sex-chromosome divided.



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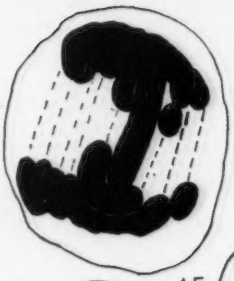

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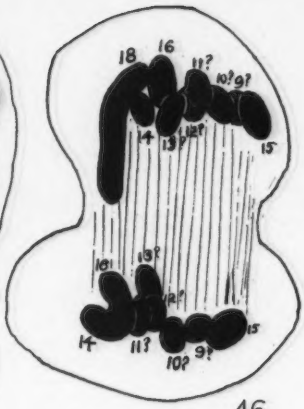

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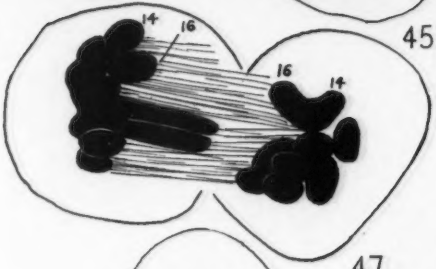

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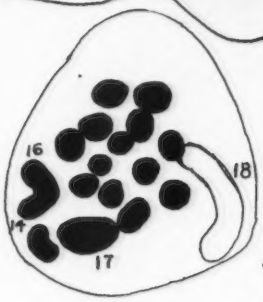

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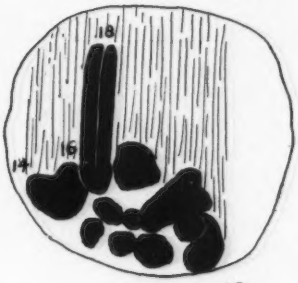

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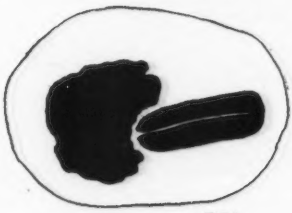

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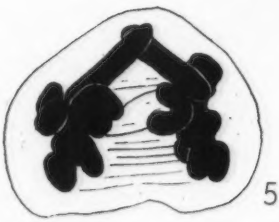

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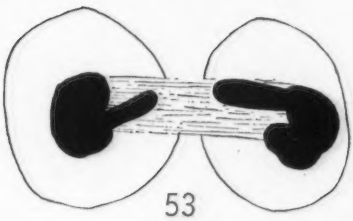

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PLATE VI.

Jamaicana unicolor n. sp. Caudell.

Spermatogonial figures of two members of this species. Thirty-five rods are found in the one; thirty-one rods and two V's are found in the other. Figures 65–67 are first spermatocytes.

FIGS. 54–57. Spermatogonial figures of individual 430. There are thirty-five chromosomes of the rod type here, paired and numbered according to size. No. 18 is the unpaired sex chromosome.

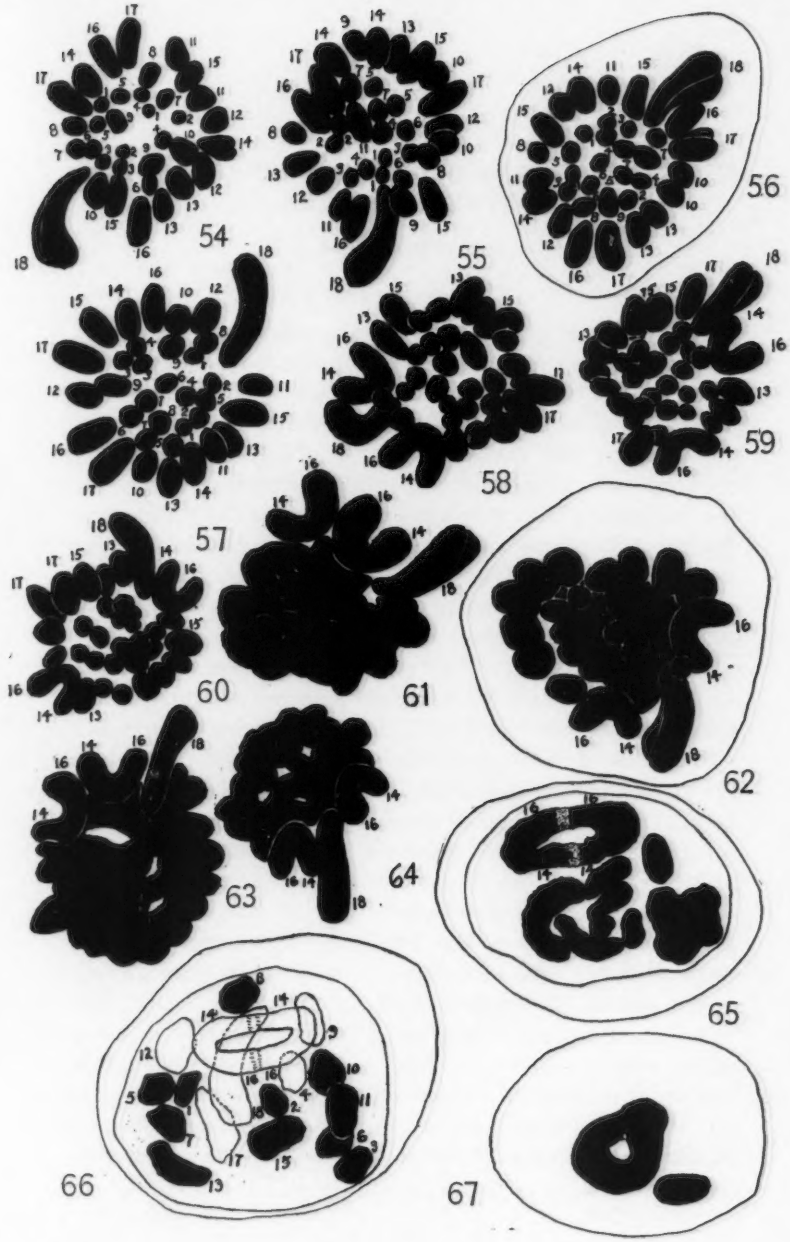
FIGS. 58–60. Spermatogonial figures of individual 560 showing thirty-one rods and two V's in each. No. 18 is the unpaired sex-chromosome. Just the largest chromosomes are paired and numbered.

FIGS. 61–64. Spermatogonial metaphases. Although the chromatin material is much massed, the sex and the two V-chromosomes are very prominent and distinct.

FIG. 65. First spermatocyte of individual 560 showing the multiple or bi-tetrad formed by the two V's.

FIG. 66. First spermatocyte prophase showing the complete number of chromosomes in individual 560,—fifteen rod tetrads, the bi-tetrad, and the sex-chromosome.

FIG. 67. The ring-shaped bi-tetrad more condensed in a latter prophase.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PERIODICITY IN THE PRODUCTION OF MALES IN HYDATINA SENTA.[1]

A. FRANKLIN SHULL.

INTRODUCTION.

There is often a well-marked rhythm in the production of males in the rotifer *Hydatina senta*. Generation after generation may pass with few or no male-producing females; while later, in a few successive generations, male-producers may be abundant, only to be succeeded by a period in which male-producers are uncommon or wanting. Although this rhythm has not been mentioned by all students of the life cycle of *Hydatina*, and has been emphasized by few of them, it can hardly have escaped notice by any one who has bred this species for several months. In another genus, *Asplanchna*, Mitchell (1913) has laid stress upon this rhythmical appearance of males, as a basis for certain theoretical conclusions, and has called attention, by way of generalization, to the same rhythm in *Hydatina*.

Regarding the cause of this periodicity, there is not general agreement. Mitchell, writing of *Asplanchna* but extending his conclusions to rotifers in general, appears at times to regard the rhythm as the effect of an internal factor, and again as due to environmental conditions. He says "this rhythm is not the result of external conditions" but "is not absolutely independent of them."[2] Later he adds that "male production . . . is a matter of physiological potential and under the more or less direct control of nutrition" (p. 229). At other places he states that "male production . . . is a phenomenon all but wholly under nutritive control . . ." (p. 246); "these male and non-male producing strains . . . exist . . . and . . . these strains are also produced by nutritive changes" (p. 247); and "qualitative and quantitative changes in nutrition will be found the universal sex-controlling factors in this group" (the rotifers)

[1] Contribution from the Zoölogical Laboratory of the University of Michigan.

[2] *Op. cit.*, p. 228.

(p. 253). Whitney (1914), on the other hand, has no hesitancy in ascribing the periodicity of male production to external factors; thus, in mentioning earlier work of his own, "he was of the opinion that whatever the potent factor was that sometimes caused only females to be produced, and at other times caused nearly all males to be produced, it must be an external factor." On the basis of his recent work he attributes this periodicity to alternation of the active and quiescent states of the protozoan food of the rotifers.

In my own work on *Hydatina* during several years, certain lines have been bred so long, and so many families completely reared, that further light may be thrown upon the rhythm of male production. In the following pages evidence is first presented, bearing on the regularity of the periodicity of male production, and the probable independence of this periodicity of the environment. Later the supposed evidence that male production is correlated with nutrition is discussed.

Regularity of the Periodicity of Male Production.

Conditions Necessary to the Demonstration of Periodicity.

In parthenogenetic lines that produce many males, rhythm is not as easily demonstrated as in lines producing few males. If the proportion of males in a line producing many males be represented by a curve, there are so many irregularities in it, so many minor humps even in the periods of few males, that the larger humps are less striking. If the lines produce few males, on the other hand, the whole curve may be so lowered that the periods of depression are below the base line; that is, there are no males at all in these periods. In such cases the rhythm may be quite striking.

Further difficulty in detecting rhythm is introduced by rearing only one family in each generation. There are great individual differences between families taken at the same period, so that the family chosen may or may not be an average of all families that might be reared at the same time. Furthermore, the method of publishing the results, merely giving the total for the one family in each generation, often makes the rhythm appear less definite; because it often happens that in three or four successive

generations, in a period of many males, the males are produced mostly by the daughters at the end of the family in the first of these generations, by the daughters in the middle of the family in the second generation, and by the daughters in the first part of the family in the third generation. In the manner in which these results have been published, the males of the first and third generation (appearing under date of the beginning of their respective families) seem to be four or five days apart, whereas they may be only one day apart, or even hatched on the same day.

It seemed advisable to obviate as many of these difficulties as possible, and to obtain evidence of periodicity less open to objection. The method adopted was as follows: First, a line was selected which was producing only a moderate number of males, with the expectation that the periods of male production would be completely separated by periods in which there were no males at all. Second, instead of isolating all the daughters of a single family in each genertion, parts of a number of families were isolated. This second precaution was taken to smooth out irregularities and to reduce the *apparent* length (and hence the overlapping) of the periods, both of many and of few males.

Lines Exhibiting Periodicity.

The line selected to meet the above conditions was one of the F_2 lines from a cross between an English and a Nebraska line, described in an earlier paper (Shull, '15). Several females of this line, all of approximately the same age, were placed together in a dish. When they reached maturity, the daughters of the early part of their families were rejected; but the daughters produced after about 24 hours of egg laying were preserved. From these daughters the "sex ratio" (ratio of male-producing to female-producing females) was determined, and from them also the next generation was reared. The first daughters were in each case rejected because, as I have shown before (Shull, '10), the first daughters are less commonly male-producers than are daughters in the middle of the family.

The daughters selected for rearing the next generation were in like manner kept together in one dish until about 24 hours after their first daughters appeared. At this time a second lot, all of

about the same age, was isolated to determine the sex ratio of the second generation, and so on.

From 30 to 100 daughters, all of nearly the same age, and from the same parts of their respective families (end of first day of egg-laying) were recorded for each generation. A new generation was, by this method, secured every three days, as a rule, instead of every two days, as is possible when the first daughters of a family are used as parents of the next generation.

The number of male-producing and female-producing females in each generation thus recorded is given in Table I. Male pro-

TABLE I.

SHOWING THE NUMBER OF MALE-PRODUCING (♂♀) AND FEMALE-PRODUCING (♀♀) FEMALES IN A LINE OF *Hydatina senta*.

The male-producers occur periodically.

Date.	Number of ♂♀.	Number of ♀♀.	Date.	Number of ♂♀.	Number of ♀♀.
Jan 16	2	24	Mar. 14	0	44
19	0	24	17	0	38
22	1	35	20	0	42
25	3	33	23	0	23
28	19	46	26	8	40
30	14	46	29	36	48
Feb. 2	0	56	Apr. 1	7	71
5	0	40	4	1	51
9	2	38	7	0	37
12	0	43	10	0	40
15	0	47	13	1	36
18	0	48	16	0	35
21	0	13	19	0	43
24	0	35	22	3	85
27	17	21	26	68	51
Mar. 2	13	27	29	32	23
5	6	29	May 2	18	34
8	2	36	5	3	50
11	0	42	8	0	44

duction in this line, when bred as described above, showed a well-marked periodicity, the interval being about one month. Conditions were purposely kept fairly uniform; but even if it were not possible to prevent changes of the medium, it is scarcely probable that any external condition favoring male production should have recurred with such regularity. Moreover, were this periodicity due to external factors, the intervals between periods of male production should be the same in all lines bred at the same time and subjected to the same conditions. That this was not the case will now be shown.

In another line, bred for a much longer time than was the line described above, there was equally clear evidence of regular periodicity, though the extent of the waves of male production was not determined. A line of rotifers obtained from England in the fall of 1912, described in another paper (Shull, '15), has been reared up to the present time. No considerable numbers of individuals of this line have as a rule been *isolated*, hence the sex ratio can not be stated; but during the time when the periodicity of other lines was being examined, several dozen

TABLE II.

SHOWING DATES BETWEEN WHICH MALES WERE PRODUCED IN AN ENGLISH LINE OF *Hydatina senta*.

No males appeared between one period of male production and the next.

Number of Period of Male Production.	Dates Between Which Males Were Found.
First	February 13 to 25, 1913
Second	April 17 to 26, 1913
Third	June 19 to 24, 1913
Fourth	August 24 to 30, 1913
Fifth	October 31 to November 15, 1913

individuals of most generations were reared in two or three dishes. As the number of males was always small in this line, males were found in these "mass" cultures only occasionally. They nearly always appeared in the dishes containing several successive generations, and then were wanting for a considerably longer period. There was thus a rhythm of male production, which, as shown in Table II., proved to be fairly regular and definite, though the number of male-producers in each period was not known.

The interval between periods of male production was, in this line, a trifle over two months. Inasmuch as this line was bred in part simultaneously with the one recorded in Table I., the difference in the interval of male production in the two lines (one month in the first, over two months in the second) effectually disposes of any suspicion that this rhythm was induced by external conditions.

A third line which showed evidence of periodicity in the production of males was obtained from Nebraska in 1912, and has been reared ever since. This line is also described in a recent

paper (Shull, '15). Complete families were isolated from early in November to December 15, 1912, but no males appeared. During the time when periodicity of male production was being studied, representative mass cultures were reared. These cultures showed males in small numbers only, and at times separated by wide intervals. The times of male production, as far as known, are shown in Table III., though the proportion of males was not recorded.

The periods of male production in the Nebraska line are separated by intervals of three to five months, the interval

TABLE III.

SHOWING TIMES AT WHICH MALES WERE PRODUCED IN A NEBRASKA LINE OF *Hydatina senta*.

Number of Period of Male Production.	Dates Between Which Males were Found.
First	January 15 to February 1, 1913
Second	April 22, 23, 1913
Third	August 10 to 16, 1913
Fourth	December 7 to 11, 1913
Observation wanting[1]	
Sixth(?)[2]	November 1, 1914

increasing with the age of the line. Though no complete families were reared, during any of these "waves" of male production, it was evident that in the later periods there were fewer males than in the earlier ones.[3]

In each of the three lines described, there was a well-marked rhythm in the production of males. The great regularity of this periodicity, especially in the first two of these lines, and the fact that the lines differed considerably from one another in regard to the interval between periods of male production, forbid the assumption that the waves of male production were brought on by specific external conditions.

[1] Observations were wanting from March 8 to June 10, 1914. Probably not more than one period of male production fell between these dates, hence the next males recorded are to be regarded as belonging to the sixth period.

[2] A single male-producing female appeared in this "wave" of male production.

[3] In the English line described in Table II., while the intervals between periods of male production did not increase, with the age of the line, the number of males in successive periods plainly decreased. This is a confirmation of a conclusion which I formerly drew from lines bred through shorter periods, namely, that the proportion of male-producing females gradually decreases with the age of the line. Whether, as Mitchell suspects, this decrease is due to uniformity of conditions, is a question not answered by the evidence.

No statement here made is to be construed, however, as a contradiction of my former claim that external conditions may alter the *extent* of male production.[1] Few biological facts are more firmly established than that external factors modify the life cycle of *Hydatina*. The results described above merely show that, under fairly uniform conditions, there is nevertheless a periodicity in the production of males which must be due to internal factors.

Periodicity and Nutrition.

Mitchell ('13) has pointed out that in *Asplanchna* periods of male production are also often, perhaps usually, periods of vigorous growth and rapid reproduction; and he concludes therefrom that male production is a result of high nutrition. This conclusion may be correct, but it is scarcely logical, since coincident events are not always related to one another as cause and effect. But assuming as Mitchell does that size of family is a guide to nutrition, let us examine all the sources of information that are extensive enough to be of value, to determine, if possible in this way, the relation of nutrition to male production. In my own work in the past few years, there have been two lines in which hundreds of families have been reared. By collecting all of the families of the same size in a single line, and recording the proportion of male-producers, it should be possible to discover to what extent size of family and male production are correlated. Obviously one must not collect in the same group families belonging to two or more unrelated lines, for one of these lines may have larger families, and at the same time (but from other causes) either many or few male-producers, so that the groups of families of large size would have on the average a correspondingly high or low proportion of male-producers. Such an apparent correlation would have no significance. Within a single line, however, no such error could affect the results. The two tables herewith presented (IV. and V.) are each compiled from families belonging to a single line.

[1] My discovery several years ago of internal differences between parthenogenetic lines of *Hydatina senta*, the result of which is a different proportion of male producers in each line, is characterized by Mitchell as a "return to the position of Punnett." Since Punnett never found an effect of external conditions, and since I never repudiated my experiments proving the effect of external conditions, there can have been no "return."

Notwithstanding the great fluctuation of the proportion of the male-producing females in families of different sizes, it might be possible to see in the percentages given in the last columns of these two tables (or perhaps only Table IV.), a slight increase from top to bottom, and hence a correlation between size of family and male production; though the degree of correlation

TABLE IV.

SHOWING SIZE OF FAMILY AND PROPORTION OF MALE-PRODUCING (♂♀) AND FEMALE-PRODUCING (♀♀) FEMALES IN A SINGLE PARTHENOGENETIC LINE OF *Hydatina senta*.

Size of Family.	Number of Families.	Number of ♂♀.	Number of ♀♀.	Percentage of ♂♀.
1 to 5	7	3	13	18.7
6 to 10	15	15	96	13.5
11 to 15	16	47	158	22.9
16 to 20	20	28	322	8.0
21 to 25	20	73	388	15.8
26 to 30	21	152	437	25.8
31 to 35	33	174	909	16.1
36 to 40	27	213	816	20.7
41 to 45	29	281	977	22.3
46 to 50	26	324	931	25.8
51 to 55	19	285	710	28.6

certainly can not be high. However, even if such correlation exist, it does not follow that the percentage of male-producing females is dependent upon the degree of nutrition, which determines size of family. The one way to test the effect of nutrition is to alter it artificially, and note the results. That has been done in my starvation experiments (Shull, '10, pp. 320 ff.), with results that were positive but of such a character that they could be explained as due to changes in the chemical composition of the medium, rather than changes of nutrition. Mitchell ('13) objects that in drawing conclusions from these starved families, I have regarded only the totals; had I observed individual families, he believes, I would have reached a different result. Mitchell states that the male-producing females in my starvation experiments did not appear in the smallest families, which were presumably the offspring of the most starved parents, but in the larger families, produced by the better nourished females; and from this supposed fact concludes that abundant male production is due in *Hydatina*, as he believes it to be due in *Asplanchna*, to

high nutrition (coupled with irregularities of nutrition). How far his statement that the male-producing females are in the larger families is correct, may be seen from Table VI., in which

TABLE V.

SHOWING SIZE OF FAMILY AND PROPORTION OF MALE-PRODUCING (♂♀) AND FEMALE-PRODUCING (♀♀) FEMALES IN A SINGLE LINE OF *Hydatina senta*, DISTINCT FROM THAT IN TABLE IV.

Size of Family.	Number of Families.	Number of ♂♀.	Number of ♀♀.	Percentage of ♂♀.
1 to 5	12	2	38	5.0
6 to 10	17	13	121	9.7
11 to 15	19	67	183	26.8
16 to 20	29	105	415	20.1
21 to 25	32	152	587	20.5
26 to 30	17	48	418	10.3
31 to 35	22	149	577	20.5
36 to 40	15	113	463	19.6
41 to 45	18	76	704	9.7
46 to 50	19	192	716	21.1
51 to 55	8	106	314	25.2

the families of the starved line in the experiment referred to (Shull, 1910, Table III.) are tabulated. The group of families containing 1 to 5 daughters, and that of families numbering 41 to 45, are omitted because there is but one family in each group.

TABLE VI.

SHOWING SIZE OF FAMILY AND PROPORTION OF MALE-PRODUCING (♂♀) AND FEMALE-PRODUCING (♀♀) FEMALES IN A STARVED LINE OF *Hydatina senta* DESCRIBED IN A FORMER PAPER.

The greatest proportion of male-producers is in families of medium size.

Size of Family.	Number of Families.	Number of ♂♀.	Number of ♀♀.	Percentage of ♂♀.
6 to 10	7	7	49	12.5
11 to 15	3	6	34	15.0
16 to 20	6	39	66	37.1
21 to 25	8	88	101	46.5
26 to 30	16	172	273	38.6
31 to 35	8	96	169	36.2
36 to 40	2	14	64	17.9

In this table it appears that the greatest number of male-producing females is found, not in the largest families, but in those of medium size. It may also be recalled that the distribution of the male producers with regard to size of family, in these starved families where nutrition was *known* to have been variable,

is not the same as the distribution in the well-fed families of Tables IV. and V., about whose nutritive conditions we know only that which size of family tells us. The argument that the numerous male-producing females of the starved line were produced as a result of high nutrition of their parents, loses much of its weight when it is shown that these male-producers were not chiefly in the largest families.

It is not to be asserted that nutrition has no effect upon male production. Indeed, Whitney ('14) has presented new evidence that *qualitative* differences of nutrition do affect male production. It is not clear what relation Whitney's results have to the question of *periodicity* of male production, whether changes of nutrition can be made to destroy the rhythm, or wholly to alter the interval, or merely to modify the extent of male production. My own starvation experiments, referred to above, left the intervals between the periods of male production unaltered, but the waves of male production and the intervening periods of female production were rendered less striking. I attributed the effects shown in these experiments to the chemical nature of the medium, and not to nutrition. Until experimental evidence indicates the contrary to be true, it is safest to assume that nutrition also, when it affects male production at all, does not alter the interval between periods of male production, but merely the extent of male production.

To summarize: Three lines of *Hydatina*, bred through many months, showed fairly regular periodicity in the production of males. One line exhibited relatively abundant male production every month; another every two months; while in the third the interval varied from three to five months during the period of observation. The fact that the interval between the periods of many males is quite regular in some lines, and is not the same in all lines reared simultaneously, indicates that this periodicity is due to an internal factor. Hundreds of families were examined to determine whether the largest families, which were presumably offspring of the best nourished parents, contained the greater number of male producing females, as Mitchell assumes they do. In well nourished lines there is some doubt whether there was any correlation between size of family and number of male

producers; in starved families, on the other hand, the greatest numbers of male producers were not in the largest families, but in those of medium size. On the statistical evidence as a whole, the influence of quantity of nutrition upon male production is held to be "not proven." When qualitative differences in nutrition affect male production, the interval between periods of many males probably remains unchanged.

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NOTE ON TREMATODE SPOROCYSTS AND CERCARIÆ IN MARINE MOLLUSKS OF THE WOODS HOLE REGION.[1]

EDWIN LINTON.

In the summers of 1909 and 1910, while engaged in the study of the parasites of fishes at the laboratory of the Bureau of Fisheries, Woods Hole, Mass., I examined a number of invertebrates for larval stages of trematodes. The results of these examinations, although rather meagre with respect to the number of species found, are not without interest.

The following species of mollusks were examined: *Crepidula fornicata*, *C. plana*, *Ilyanassa obsoleta*, *Littorina littoria*, *L. rudis*, *Modiolus plicatulus*, *Mya arenaria*, *Mytilus edulis*, *Neverita duplicata*, *Pecten irradians*, *Purpura lapillus*, *Urosalpinx cineria*, *Venus mercenaria*. Besides these several species of crustaceans were examined, also one annelid, *Hydroides dianthus*.

Larval trematodes were found in only two species of mollusks, viz. *Ilyanassa obsoleta* and *Pecten irradians*. No trematode parasites were found in any of the crustaceans. A sporocyst found in the annelid, *Hydroides dianthus*, has already been reported.[2]

1. Sporocysts and Cercariæ from Ilyanassa obsoleta. Figures 1–6.

Snails of this species were examined on six occasions. In all but one of these examinations sporocysts were found. On each occasion a considerable number of the snails were examined with much care, the several organs being teased under a lens. None of the sporocysts, however, were seen in place, in all cases having been found lying at the bottom of the dish in which the snails had been dissected.

The following extracts from my notes made at the time of

[1] Published by permission of Commissioner of Fisheries.

[2] Biological Bulletin.

collecting will give details of frequency of occurrence with such other observations as seem to be appropriate.

1909. July 3, 34 snails examined, no parasites found.

July 19, 110 snails examined, 21 sporocysts found. The sporocysts were inactive and contained tailless cercariæ which were very active. So far as examined the sporocysts in this lot

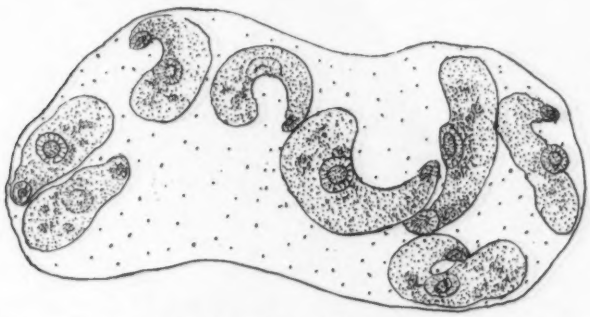

FIG. 1. Sporocyst containing cercariæ, from *Ilyanassa obsoleta;* in sea water flattened under cover glass. Length 0.86 millimeter.

contained relatively few cercariæ, 9 being the greatest number seen, and as few as 2 noted in one sporocyst.

Three sporocysts lying free in sea water had the following dimensions in millimeters:

Length	0.62	0.75	0.88
Breadth	0.30	0.26	0.30

Two cercariæ, killed under cover-glass over flame, had the following dimensions:

Length	0.31	0.42
Breadth, anterior	0.05	0.07
middle	0.13	0.14
posterior	0.06	0.06
Anterior sucker	0.04	0.05
Ventral sucker	0.07	0.06

July 20, 120 snails from North Falmouth. A dozen or more of these were dissected under a lens in the endeavor to find exactly where the sporocysts occur but without finding any in place. All the snails were then picked to pieces, washed, and the

water decanted. About 100 sporocysts were obtained. These lay motionless on the bottom of the dish. They were easily seen on a black background, being whitish translucent. They were short oblong with rounded ends, often slightly arcuate.

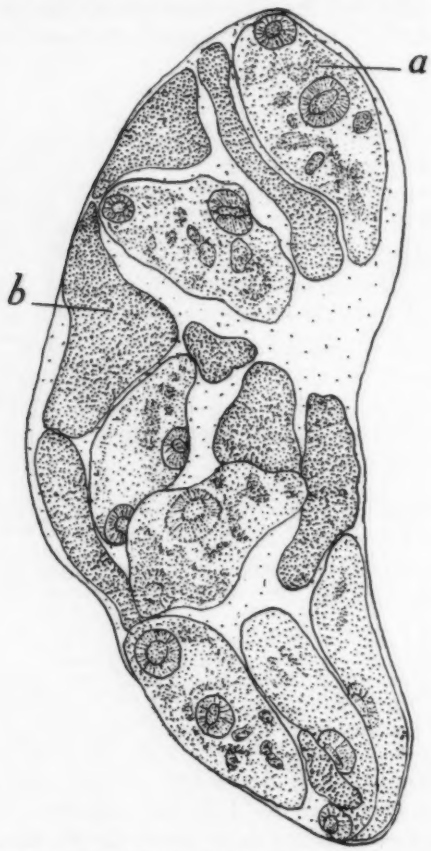

FIG. 2. Sporocyst from *Ilyanassa obsoleta*, flattened under cover glass, fixed over flame, stained and mounted in balsam. *a*, cercariæ. *b*, germinal cell masses and young stages of cercariæ. Length of sporocyst 0.80 millimeter.

July 22, about 300 snails from Tarpaulin Cove were broken open and about 80 of them removed from their shells. A few of these were looked over carefully, much of the material being

teased and examined with the aid of the compound microscope. No sporocysts were seen in place. Sporocysts were found on the bottom of the dish in which the snails that had been removed from their shells were lying, also in the dish which held the snails still in the broken shells. It would appear that the sporocysts

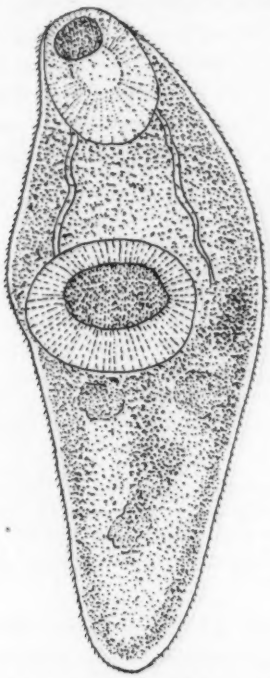

FIG. 3. Cercaria in sea water, ventral view, showing excretory vessels in front of ventral sucker, rudiments of testes, etc. Length 0.25 millimeter. From *Ilyanassa obsoleta*.

are rather loosely lodged in the mantle cavity, since they make their appearance when the broken shells and partly exposed animals are shaken about in the water. Some 600 sporocysts were obtained from this lot in a short time in this way. When the snails were picked to pieces, washed, and the water decanted, an increased number of sporocysts were obtained. The number of cercariæ in these sporocysts was greater than that recorded in the

lot collected on July 19. One sporocyst when opened liberated 40 cercariæ. Some of these were immature. An anterior spine was noted for the first time on these cercariæ. It is embedded in the tissues of the head and may be seen protruding its sharp tip at the extreme anterior end in certain stages of extension while the cercaria is actively contracting. What were taken to be excretory vessels were seen extending from near the lateral margins of the oral sucker. They appear to unite in front of the oral sucker and again behind the ventral sucker. There was a large and conspicuous excretory space near the posterior end which communicated with the terminal pore by a slender canal.

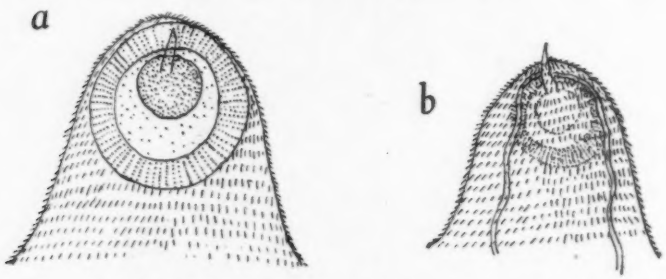

FIG. 4. Free-hand sketches of anterior end, life; showing oral spine, minute spines on surface of body, and anterior excretory vessels. *a*, dorsal view. Diameter of oral sucker 0.04 millimeter. *b*, Ventral view of another specimen. Diameter of oral sucker 0.06 millimeter; length of oral spine 0.017 millimeter. From *Illyanassa obsoleta*.

A cercaria, flattened slightly and fixed over the flame, had the following dimensions in millimeters: Length 0. 24, breadth 0. 10, oral sucker 0.041, ventral sucker 0.057, length of anterior spine 0.020. The posterior end was truncated as if slightly retracted.

July 23, 50 snails from a small salt water pond were removed from their shells and carefully dissected; about 350 others were broken open and stirred about vigorously. After a careful search 14 sporocysts were found. The pond from which these snails came, while salt, did not have free communication with the sea.

In 1910 a lot of snails that had been kept in a dish of sea water for several days were opened on different dates with the following results:

August 24, 24 snails were removed from their shells, picked to pieces with forceps, washed, the water decanted, and about 10 sporocysts found. These were linear oblong, frequently arcuate and slipper shaped, thickish. Dimensions in millimeters:

Length	0.66	0.56	0.60	0.56	0.70	0.55
Breadth	0.25	0.26	0.25	0.25	0.33	0.25

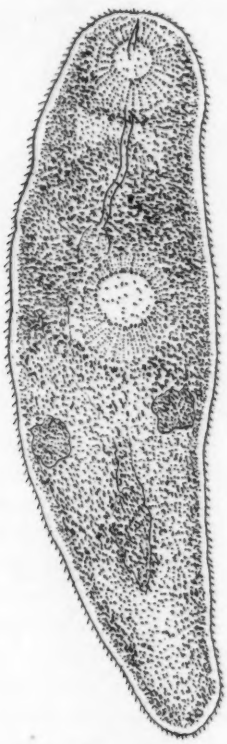

FIG. 5. Cercaria from *Ilyanassa obsoleta*, stained and mounted in balsam, dorsal view, showing oral spine, rudiments of prepharynx, pharynx, testes, etc. Length 0.30 millimeter.

August 26, 24 snails were examined that had been opened and removed from their shells the day before. No sporocysts were found. Another lot of 24 were opened and examined on this date. No sporocysts were found.

August 29, 36 snails examined in the usual way. No sporocysts were found.

The following notes were made on material that had been stained and mounted in balsam.

The shape of the cercariæ varies greatly but seems to be due to

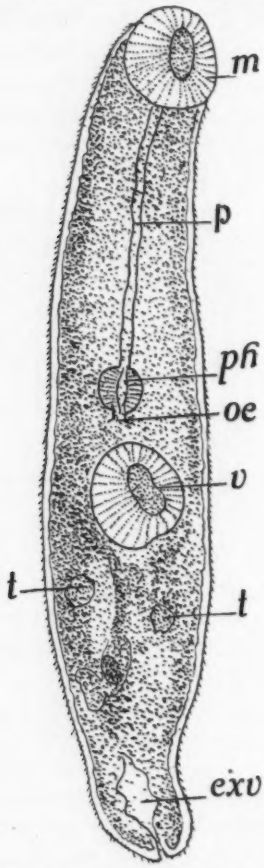

FIG. 6. Cercaria from *Ilyanassa obsoleta*, in balsam. Length 0.30 millimeter. *exv*, excretory vessel; *m*, oral sucker; *oe*, esophagus; *p*, prepharynx; *ph*, pharynx; *t*, testes; *v*, ventral sucker.

different methods of fixing, or, at least to different degrees of contraction. When fixed under pressure they are more or less

elongated. When much flattened they are usually long ovate, the greatest width being at the ventral sucker, which is near the middle of the length, thence they taper towards each extremity but more towards the posterior than the anterior end. When less compressed they may be long fusiform, or subcylindrical. The body is covered throughout with exceedingly minute spines. When the cercariæ are fixed without pressure they may be ovate, short fusiform, always thickish, frequently arcuate, the neck especially having a tendency to be bent ventrad. The suckers are nearly equal but there appears to be some variation. In

FIG. 7. Sporocyst with cercariæ, from *Pecten irradians*, life, under slight pressure; two cercariæ escaping from one end of sporocyst. Length 1.78 millimeter.

most cases the ventral sucker is slightly larger than the oral. The apertures of the suckers are variable depending on the state of contraction when fixed. Frequently the aperture of the ventral sucker is transverse. In one case it was elongated axially. The aperture of the oral sucker was in most cases nearly circular. The pharynx is subglobular and lies near the anterior border of the ventral sucker. The intestinal rami were not distinctly shown. The anterior spine is not easily seen in the mounted specimens. The stained and mounted material does not usually show more of the excretory system than the posterior vessel which is very conspicuous in the living worms. In some of the mounted specimens this posterior vessel was evident; in others it could not be distinguished. In the flattened, oval individuals it was not seen (fig. 5). In the cylindrical forms it was usually visible (fig. 6). Rudiments of reproductive organs appear in all the older cercariæ. The most conspicuous of these are two lat-

erally placed subglobular bodies which are situated a short distance behind the ventral sucker. These I take to represent the

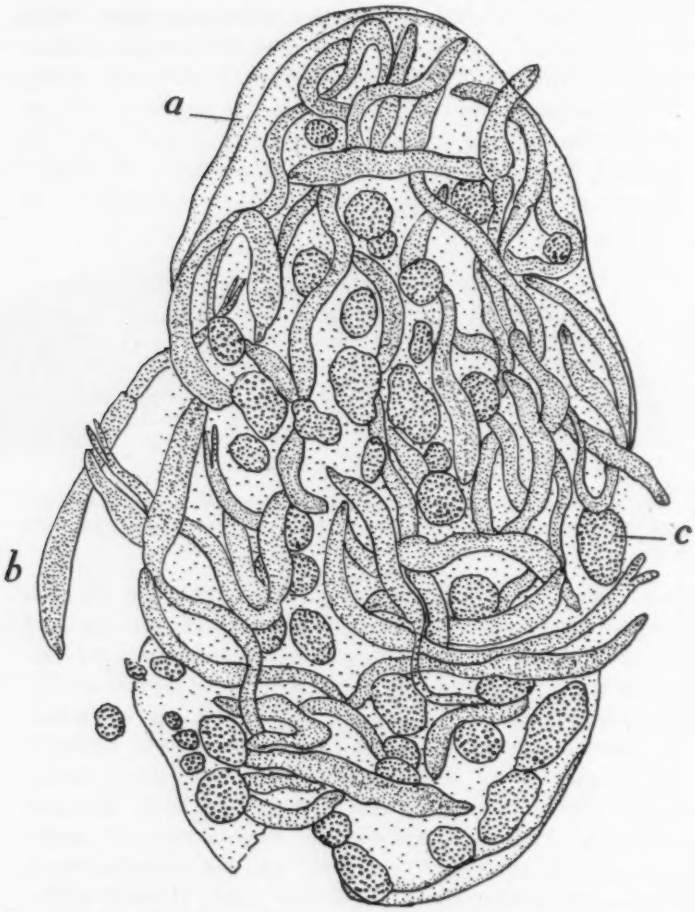

FIG. 8. Sporocyst with cercariæ, from *Pecten irradians*, stained and mounted in balsam; slightly crushed under the cover glass. *a*, wall of sporocyst; *b*, cercariæ; *c*, germinal cell masses and young stages of cercariæ. Length 0.60 millimeter.

testes.* In front of the left testis may be seen, in some, a granular mass opposite the left posterior margin of the ventral sucker

(figs. 3 and 5). This is probably the ovary. Behind the testes on the median line is a dense granular mass which is doubtless the beginning of the uterus. Granular masses which fill the body, but are most dense along the lateral margins may represent the beginnings of diffuse vitellaria.

The cercariæ of this species resemble *Cercaria linearis* Lespes, but the sporocysts are different.

2. Sporocysts and Cercariæ from Pecten Irradians. Figures 7–10.

In the summer or 1909 I examined 361 scallops on nine dates from July 3 to August 27 but found no sporocysts. In August, 1910, I examined 6 large scallops from Quisset Harbor. They had been kept in a vessel of sea water in the laboratory for two days before they were examined. After removing one valve the animals were shaken vigorously in sea water. A few small sporocysts were found in the bottom of the dish in which the scallops had been shaken. The scallops themselves were then examined carefully for sporocysts but no more were found. The sporocysts were elongate and slowly contractile with a tendency to become arcuate. The larger examples at rest in sea water measured 0.70 millimeter in length and 0.42 in breadth; length of one of the smaller specimens 0.30, breadth 0.15. A specimen compressed under a cover glass was 1.78 in length and 0.36 in breadth. These sporocysts contained numerous slender, tailed cercariæ. One of the latter in alcohol was 0.40 in length and 0.024 in breadth; another, length 0.20, breadth 0.027, length of body 0.085, length of tail 0.115. The first sporocyst examined had what appeared to be an actively contractile papilla at each extremity. These apparent papillæ proved to be cercariæ partially liberated from the sporocyst, but evidently held by the wall of the sporocyst contracting around them. All the cercariæ, both in the living and preserved specimens, are long and slender, the tail, in all cases, except immature specimens, being considerably longer than the body. In fully extended examples the tail may be two or three times as long as the body. When they are liberated from an active sporocyst they exhibit a peculiar jerking movement of the tail and posterior half of the body,

the anterior end meanwhile being bent ventrad and performing a kind of pecking movement. This characteristic behavior of the anterior end is plainly in part due to the jerking movements of the posterior portions, and in part to the alternate protrusion and retraction of a short, proboscis-like organ at the anterior end. These movements suggest adaptations to enable the cercariæ to penetrate the soft membranes of the secondary host. In some freshly liberated individuals a thin, hyaline, membranous, fin-like border was distinguished. On one of these cercariæ this membrane was observed to be broken up into slender rod-like processes which resembled long cilia. The posterior extremity is divided into two slender branches. This forked extremity was also seen to be surrounded by a thin membrane in some fresh specimens. The structure of both body and tail is coarsely granular. Rudiments of what probably represent the oral sucker, and the pharynx were distinguished. When a sporocyst is crushed, there are seen, in addition to the cercariæ, of which there may be many stages of development, some granular material and balls of cells.

While the prevailing shape of these sporocysts is long and slender considerable variation exists. Both sporocysts and cercariæ are much like those found in the annelid *Hydroides dianthus*. The cercaria resembles *Cercaria cristata* La Valette.

3. Pearls.

While the examination of numerous specimens of the edible mussel (*Mytilus edulis*) resulted negatively, so far as trematode larvæ were concerned, a few cysts were noted, some of which may have been caused by trematodes. At my request Dr. Irving A. Field, who was opening large numbers of mussels in connection with his study of the development, and experimental work on the food value of this mollusk, handed to me those that in any way appeared to be abnormal. The number of such was small.

On July 24, 1909, 2 mussels were brought to me by Dr. Field, which he thought to be in poor condition. They had been cooked, so that there was no opportunity to examine them alive. One of them had about 15 cysts from 0.5 to 2 millimeters in diameter, along the edges of the mantle, and 4 on the foot, 1.5 to

2.5 millimeters in diameter. One of these cysts when crushed proved to be filled with small granular cells irregular in outline. Besides these, 3 small pearls were found in the mantle. There were a number of small white cysts embedded in the mantle of the other mussel. These contained pearls, 58 small pearls having been obtained from them. Some of them were multiple. They measured from 0.3 to 1.12 millimeters in diameter.

On August 11 some small cyst-like yellowish masses of similar appearance to those collected on the 24th, were found on the foot and mantle of a mussel. Their contents resembled leucocytes. A smear preparation revealed round cells of different sizes, the prevailing size being about 0.01 millimeter in diameter, with very strongly staining nuclei. A very careful examination of over 100 mussels made on different dates failed to yield any parasites.

It is perhaps worthy of note that the redia stage is omitted from the larval stages of trematode development which I have found in the invertebrates of the Woods Hole region.

Reference may here be made also to another abbreviated trematode life history in the case of the distome, *Parorchis avitus*, from the Herring Gull,[1] where miracidia, still within the ova in the later folds of the uterus, contained each a single well-developed redia.

[1] *Proceedings of the U. S. National Museum*, 46: 551–555.

AN EXPERIMENTAL STUDY OF THE BEHAVIOR OF AMPHIPODS WITH RESPECT TO LIGHT INTENSITY, DIRECTION OF RAYS AND METABOLISM.

C. F. PHIPPS.

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I. Introduction.

The object of this study was to determine the effect of reagents and conditions affecting metabolism upon the reactions of amphipods to intensity and direction of light rays. The reagents and treatments used were potassium cyanide, chloretone, starvation and lowered oxygen content. All experiments were performed in the laboratory either upon amphipods just brought in, or upon those kept in captivity from one to ten weeks.

Three species of amphipods found in the vicinity of Chicago were used in the experiments, namely, a swift stream species, *Gammarus faciatus* (Say); a sluggish river and lake species, *Hyalella knickerbockeri* (Bate); and a pond species, *Eucrangonyx gracilis* (Smith).

(*A*) *Methods.*—In all the experiments a dark room was used. The special apparatus was a light grader, designed and first used by Yerkes ('02) and described with diagrams by Mast ('11, p. 61)

and Shelford ('14). In the light grader the animals are kept during experiments in a small rectangular tank having glass sides. A false bottom in this tank allows running water to pass through and thus keep the water the animals are in at constant temperature. Midway between this tank and the nernst lamp in the grader there is a partition having a triangular aperture and this aperture is covered by a lens. By this means an intense field of light is made to fall upon one end of the small tank when the latter is placed at the focal point of the lens. This field of intensity shades off to darkness in the opposite end of the tank because of the triangular opening, thus making an intensity gradient. The light which passes through the glass sides of the small tank is reflected by mirrors to a dead black wall in another part of the grader.

When ready for the experiment the animals were placed in the small tank, usually three at a time, and allowed to remain in darkness for a short time to recover from the shock of handling. Then the light was flashed upon the tank and immediately the animals were released from the glass tube with which they had been confined. Every thirty seconds the relative positions of the animals in the tank were recorded. In part of the experiments 40 readings each were taken, the first 10 of which were discarded because of the excitement of the animals due to handling and to the flashing of the intense light upon them. In the remaining experiments 25 readings each, with the first five discarded, were found to be enough to give typical results.

In each set of experiments the tank occupied by the animals was placed in three different positions: position 1, at right angles to the direction of the light rays; position 2, at an angle of 45° to the direction of rays with the *dark* end nearer to the lamp; position 3, at an angle of 45° to the direction of rays with the *light* end nearer to the lamp. The animals were first released in the field of intense light and a series of readings taken; then the same animals were released in the dark end, and oftentimes also in the place of medium light, the readings being repeated in each case.

About 50 of the 257 experiments performed were eliminated because of avoidable errors in preliminary work. The animals

were selected at random from the pan when experimented upon and the reactions under different conditions were compared.

When treating the animals with KCN and the other reagents they were kept in shallow glass dishes with a glass cover plate sealed on with vaseline to prevent evaporation. In the experiments with these animals the same solutions which they had been kept in were used in order to avoid the possibility of any stimulation which might occur by changing to tap water. The untreated amphipods were kept, and experimented on, in tap water. Filtered tap water was used in working with starved animals.

It was necessary, first, to establish a standard for the normal reactions of untreated amphipods, and then to compare with this any different reactions of treated amphipods. The series of experiments on untreated amphipods, by which the standard for normal reactions was established, form a good control series with which to compare the reactions of treated amphipods. Untreated amphipods were considered normal if they were negative to intense light when the tank was in position 1 or position 3. They were also considered normal if they were positive to intense light when the tank was in position 2. The reaction of the animals in position 2 of the tank shows that the direction of rays has a stronger influence than light intensity, for, though negative to intense light, in no case in any of the experiments with untreated animals with the tank in this position did the majority remain in the dark area. We may conclude from this that amphipods are negative to direction of rays. With treated amphipods these normal reactions were reversed in many experiments as will be shown later.

It was impossible to separate the influence of light intensity from that of direction of rays in cases where the tank was in position 3. Normally with the tank in this position both light intensity and direction of rays force the animals to the dark end. The percentage of those seeking the dark end when the tank was in this position was much greater than when the tank was at right angles to the rays, showing again the negative reaction to the direction of rays. The ray direction does not function when the tank is at right angles to it, except possibly to force

the animals to the side of the tank farthest away from the lamp. Comparative figures which will be given later indicate at least that ray direction may force the animals to the side farthest from the lamp. Possibly the data as regards light intensity *vs.* ray direction has been over-emphasized.

We can base our conclusions definitely on the effect of the direction of rays only upon reactions with the tank set in position 2. The standard for this experimental work is based therefore both upon light intensity and direction of rays.

II. Responses of Untreated Amphipods to Light Intensity and to Direction of Rays.

Table I. shows the reactions of untreated amphipods when the tank was at right angles to the direction of light rays (position 1). Not only are the three species compared but also the stock kept in the laboratory several weeks is compared with that freshly brought in.

Table I.

Untreated Amphipods.

Experimental Tank at Right Angles to Direction of Rays (Position 1).

Species.	In Captivity.	Total Readings.	Normal Reactions.				Reversed Reactions.			
			Readings.	% +.	% ∝.	% −.	Readings.	% +.	% ∝.	% −.
Gammarus.....	4 weeks	150	150	37	21	42		0	0	0
	2 days	120	60	27	19	54	60	54	26	20
Hyalella.....	4 weeks	130	130	16	7	77		0	0	0
	1 day	150	150	13	10	77		0	0	0
Eucrangonyx...	6 weeks	150	150	13	11	76		0	0	0
	1 day	60	60	20	7	73		0	0	0

+ indicates a positive reaction, ∝ an indifferent reaction, − a negative reaction.

The strongest negative reaction to intensity is shown by the *Hyalella* and *Eucrangonyx* species, 73 to 77 per cent. This means that in the 150 half minute readings, *e. g.*, *Eucrangonyx* 6 weeks stock, only 13 per cent. of the animals were found in the area of intense light, 11 per cent. in the medium or dim light and 76 per cent. in the dark area. Although 13 per cent. were in the field of intense light at the time the readings were taken, yet, in most instances, the stay in that area was but momentary. If the animals in their "random movements" or "busy explora-

tions" entered the field of intense light they were plainly stimulated and usually darted back quickly to the dark area.

The typical reaction for the species, negative to intense light, was that given by a majority of the untreated amphipods in the series of readings taken, and the group was considered to give reversed reactions only when a majority, in a series of readings, was found in the region of greatest light intensity. Such a reversal was found with one group of *Gammarus*, fresh stock, where in 60 readings the majority, 54 per cent., were found to be positive to intense light, while but 20 per cent. were negative, at the time of the readings. Another group of *Gammarus* fresh stock, however, was just as strongly negative to intensity. In this case of reversed reaction the animals remained in the intensity field much more constantly than did those of other groups which showed strong negative reactions. These few reversals with *Gammarus* may be due to the intense light, or to some factor not recognized. Mast ('11) has reported cases where long continued or increased light intensity has reversed the phototactic reactions of certain animals. The terrestrial form of amphipods, *Orchestia agilis*, is negatively phototactic when first exposed to light, but becomes positively phototactic with bright light, the stronger the light the quicker the reaction.

When the tank was placed in an oblique position so that it was at an angle of 45° to the direction of light rays with the dark end nearer the lamp (position 2), the majority of reactions in all the experiments performed were normal. As stated above, this normal reaction is based upon both light intensity and direction of rays, and as the direction of rays exerts a stronger influence than does intensity, the animals were forced to the light end of the tank, when the tank was in this position, and so appear positive to intense light. The percentages of normal reactions (positive readings), with the tank in position 2, are not so high as in position 1 (negative readings). This undoubtedly is due to the fact that the influences of intensity and direction of rays were working against each other when the tank was in position 2.

The position of the tank was again changed so that it was at an angle of 45° to the direction of rays with the light end nearer the lamp (position 3). In all except one series of readings,

namely with *Gammarus* fresh stock, the large majority of reactions were normal, that is, negative to light intensity and to direction of rays. In this one case of reversed reaction with *Gammarus* the same animals were used as in Table I., where a reversed reaction is shown. With the tank in position 3 the percentages of normal readings were much higher than is true of the other positions of the tank. This is due to the combined action of intensity and ray direction in position 3, both together forcing more animals to the dark or negative end of the tank than does intensity alone when the tank is in position 1. The average percentage is 78⅓ negative reaction for position 3 of the tank and 66½ for position 1.

In the above three positions of the tank a larger percentage of animals freshly obtained was negative both to light intensity and to direction of rays, in all cases except one series of readings, than were the animals kept in the laboratory for several weeks. Some factor or factors associated with long captivity apparently had an effect in lessening the negative responses of the amphipods to intensity and to direction of rays. Possibly the metabolic rate was depressed by laboratory conditions causing a tendency to a reversal.

Table II. shows the percentage of experiments giving normal

TABLE II.

UNTREATED AMPHIPODS.

Experimental Tank at Right Angles and at Oblique Angles to Direction of Rays (Positions 1, 2 and 3).

	No. of Experiments Performed.	No. of Readings.	Normal Reactions, Per Cent. of Experiments.	Reversed Reactions, Per Cent. of Experiments.
Laboratory stock.....	33	1,174	100	0
Fresh stock.....	27	990	85.1	14.9
Totals.....	60	2,164	93.3	6.7

and reversed reactions with all untreated amphipods, both with the tank at right angles and at angles of 45° to the direction of rays.

III. Responses of Treated Amphipods to Light Intensity and to Direction of Rays.

(*A*) *Potassium Cyanide.*—Only stock kept in the laboratory for some weeks was used in these experiments. Three different strengths of cyanide were tried, N/100,000, N/125,000, and N/150,000. Both reversed and normal responses occurred with all three. Probably the N/100,000 is not too strong for work with these animals and quicker results may be obtained with this strength. The animals were kept in the different solutions for varying lengths of time, one to nine days.

In Table III. the reactions of the three species are shown with the tank in position 2. In this case the *Hyalella* gave no majority of reversals in any series of readings, though the other two species showed strong reversals. Where the animals were exposed a longer time, as with *Eucrangonyx*, all the experiments gave a majority of reversed reactions. When the tank was in position 1 or 3 the results were very similar to those in position 2.

Table III.

Amphipods Treated with Potassium Cyanide.

Experimental Tank at an Angle of 45° to Direction of Rays with the Dark End Nearer the Lamp (Position 2).

Species.	In Captivity.	Total Readings.	Normal Reactions.				Reversed Reactions.				Time Exposed.
			Readings	%+.	%∝.	%−.	Readings.	%+.	%∝.	%−.	
Gammarus...	6 weeks	320	270	61	27	12	50	0	43	57	1–3 days
Hyalella.....	2 weeks	180	180	73	5	22		0	0	0	2–3 "
Eucrangonyx.	9½ weeks	90		0	0	0	90	26	22	52	2–9 "

(*B*) *Chloretone.*—For these experiments a solution of 0.0025 per cent. was used, which was strong enough to give a very perceptible odor of chloretone. The animals were kept in this solution from 8 to 12 days before experimenting. This length of time exposed undoubtedly was a factor in causing many more reversed reactions than with other treatments.

When the tank was in position 1 the *Gammarus* showed a much larger per cent. of reversed than normal reactions, but the other two species were all normal in the majority of readings. In the other two positions of the tank all three species had strong

reversals, and the *Hyalella* and *Eucrangonyx* gave no majority of normal reactions in any series of readings. See Table IV.

TABLE IV.

AMPHIPODS TREATED WITH CHLORETONE.

Experimental Tank at an Angle of 45° to Direction of Rays with the Light End Nearer the Lamp (Position 3).

Species.	In Captivity.	Total Readings.	Normal Reactions.				Reversed Reactions.				Time Exposed.
			Readings.	% +.	% ∝.	% −.	Readings.	% +.	% ∝.	% −.	
Gammarus...	8–9 weeks	150	60	35	10	55	90	56	17	27	8–12 days
Hyalella.....	4 "	60		0	0	0	60	95	2	3	9 "
Eucrangonyx.	1½ "	60		0	0	0	60	57	7	36	9 "

(*C*) *Starvation.*—Amphipods from each habitat were kept in filtered tap water and starved from 4 to 14 days before experimenting. Reversed reactions occurred with all three species, some after short treatment, others only after long treatment. Table V. shows reversed reactions with *Hyalella* and *Eucrangonyx* when the tank was in position 2. *Gammarus* gave reversed reactions with the tank in positions 1 and 3, but the majority were normal with the tank in position 2.

TABLE V.

STARVATION TREATMENT.

Experimental Tank at an Angle of 45° to Direction of Rays with the Dark End Nearer the Lamp (Position 2).

Species.	In Captivity.	Total Readings.	Normal Reactions.				Reversed Reactions.				Time Exposed.
			Readings.	% +.	% ∝.	% −.	Readings.	% +.	% ∝.	% −.	
Gammarus...	6½ weeks	60	60	56	19	25		0	0	0	4 days
Hyalella.....	7 "	120	60	71	5	24	60	38	21	41	6– 9 "
Eucrangonyx.	1½–3 "	120	60	40	21	39	60	32	17	51	5–14 "

(*D*) *Low Oxygen Content.*—The oxygen content of tap water was reduced by using a machine, devised by Shelford and Allee ('13), for deaërating water by a process of heating and then cooling to the required temperature. The oxygen content was reduced in some experiments to as low as 0.79 c.c. per liter. The dish containing the amphipods was filled with this low oxygen water and the cover sealed down with vaseline. Amphipods began to die after an exposure of about one day to low

oxygen content of from 0.79 c.c. to 1.51 c.c. per liter. Only *Gammarus* gave a majority of reversed reactions in any series of readings (see Table VI.), and this took place with the tank in positions 1 and 3. The other two species gave no majority of reversed reactions in any position of the tank.

TABLE VI.

LOW OXYGEN CONTENT.

Experimental Tank at Right Angles to Direction of Rays (Position 1).

Stock.	Total Readings.	Normal Reactions.				Reversed Reactions.				Time Exposed.	C.c. of O_2 per Liter.
		Readings.	% +.	% ∝.	% −.	Readings.	% +.	% ∝.	% −.		
Gammarus...	120	60	0	5	95	60	67	11	22	19 hrs.	0.79
Hyalella.....	60	60	34	18	48		0	0	0	17 "	1.51
Eucrangonyx.	60	60	23	18	59		0	0	0	22½ "	0.99

In Table VII. the percentage of experiments is given showing normal and reversed reactions with all treated amphipods, both with the tank at right angles and at angles of 45° to the direction of rays.

TABLE VII.

TREATED AMPHIPODS.

All Three Species. Tank in Positions 1, 2 and 3.

Treatment.	No. of Experiments Performed.	No. of Readings.	Normal Reactions, Per Cent. of Experiments.	Reversed Reactions, Per Cent. of Experiments.
Potassium cyanide.....	54	1,520	72.3	27.7
Chloretone.....	27	810	44.5	55.5
Starvation.....	29	900	69.0	31.0
Low oxygen.....	22	720	68.2	31.8

Table VIII. shows that the percentage of reversals in the three species is quite different under the same treatment. Also, in the same species, the percentage of reversals varies for each reagent used.

TABLE VIII.

TREATED AMPHIPODS.

Reversed Reactions only.

	Gammarus, Per Cent. of Experiments.	*Hyalella*, Per Cent. of Experiments.	*Eucrangonyx*, Per Cent. of Experiments.
Reversed reaction with KCN.....	39.3	5.5	33.3
" " " Chloretone....	60.0	66.6	33.3
" " " Starvation....	66.6	18.2	25.0
" " " Low Oxygen...	36.3	50.0	0.0

IV. Sides of the Experimental Tank, in Relation to the Lamp, Occupied by the Amphipods.

At each reading, when the position of the animals in relation to intensity was taken, their position in relation to the sides of the tank was also taken. The object was to determine, if possible, whether the direction of rays influenced the animals to seek the side of the tank farthest from the lamp.

The results are shown in Table IX. The readings have no reference to the dark end of the tank. In positions 1 and 2 the largest per cent. of the animals was found on the side of the tank farthest from the lamp, while in position 3 the majority were found on the side nearest the lamp. This is not conclusive, however, and these lateral positions in the tank may be due only to "random excursions" or "busy explorations" that Holmes ('01) speaks of as characteristic of active animals. Possibly the animals were reacting towards their own shadow, in positions 1 and 2, rather than to ray direction. It is interesting to note that there is very little difference in the results between treated and untreated amphipods.

In none of the experiments was there evidence of orientation either to light intensity or to direction of rays.

Table IX.

Tank at Right Angles to Direction of Rays (Position 1).

	Side Farthest from Lamp.	Median Position.	Side Nearest to Lamp.
Untreated Amphipods.....	48.5%	11.3%	40.2%
Treated "	49.2	14.9	35.9

Tank at Angle of 45° to Direction of Rays with Dark End Nearer the Lamp (Position 2).

Untreated Amphipods.....	52.9%	7.7%	39.4%
Treated "	49.2	12.6	38.2

Tank at Angle of 45° to Direction of Rays with Light End Nearer the Lamp (Position 3).

Untreated Amphipods.....	35.7%	11.5%	52.8%
Treated "	37.9	11.7	50.4%

V. Conclusions and Discussion.

(*A*) *Summary.*—1. In an experimental tank set at right angles to the direction of light rays and graded from intense light to darkness, pond, stream and river amphipods, as a group, seek the dark area, therefore are negative to light intensity.

2. When the same tank is set obliquely, at an angle of 45°, to the direction of rays with the dark end nearer the lamp, the amphipods are forced to the light area, even though they are negative to intense light. The stimulus of the direction of rays, to which the amphipods react negatively, has a stronger effect than the stimulus of light intensity.

3. If the experimental tank is set at an angle of 45° to the direction of rays so that the light end is nearer the lamp, normal amphipods, as a group, seek the dark end. In this case the direction of rays exerts the same stimulating effect as light intensity in forcing the animals to the dark area. This again shows a negative reaction to intensity and to direction of rays. A larger percentage show negative reaction, with the tank in this position, than when the tank is at right angles to the light rays.

4. When treated with certain depressing agents many of these amphiphods become reversed in their reactions to light intensity and to direction of rays.

5. Freshly obtained amphipods give a larger percentage of negative reactions, both to intensity and direction of rays, than do amphipods which have been kept in the laboratory for some time.

6. In these experiments there is no evidence of orientation of amphipods either to light intensity or to direction of rays.

7. Changes in the metabolic processes (physiological states) of the amphipods were undoubtedly the cause of reversed reactions in this series of experiments.

Some of the above results have been obtained also by other investigators. Holmes ('01) says that all aquatic amphipods studied by him were negatively phototactic, although three species of land amphipods studied were positively phototactic. Loeb ('04), writing about experiments on *Gammarus pulex* and other animals, says that "whatever increases the activity tends to increase the positive reaction to light, while anything which

tends to quiet the animals tends to make them negative." He adds also that the *Gammarus pulex*, which is negative to light, can be made positive by adding to the water a little carbon dioxid, hydrochloric, oxalic or acetic acid, ether, chloroform, paraldehyde, alcohol, esters and all ammonium salts. Boracic acid, according to Loeb, does not reverse these amphipods, but Jackson ('10), in repeating Loeb's experiments, but using *Hyalella knickerbockeri*, found that a saturated solution of boracic acid does cause a reversal, the same as the other reagents. Jackson also found that some other acids and some alkalies produce the same effect. These reversals took place, however, only when he dropped the animals into the solution, for when he put the animals into distilled water and gradually added the chemicals no reversals took place.

McCurdy ('13) says that "sunlight modifies the normal physiological changes taking place in protoplasm, checking some of the processes and probably accelerating the others. A starfish in the light moves to the shade because of disturbance by light of his metabolism." A part of this disturbance was due to there being "less CO_2 given off by the starfish when it was put in the sunlight."

(*B*) *Metabolism, Physiological States and Reactions.*—Before answers can be given to many questions that arise, much more work along these lines must be done. Other methods and treatments must be used, such as high oxygen content, caffein, acids, alkalies, carbon dioxid content, etc.

From the data obtained from this series of experiments it is evident that the responses of aquatic amphipods, like those of many other animals experimented upon, are related directly to the physiological state or condition of the animal. Anything which disturbs the rate of metabolism of the animal alters the response to stimuli. Allee ('12) with isopods, Child ('10) with planaria, Wodsedalek ('11) with may-fly nymphs, and other investigators have found this to be true.

In these experiments on amphipods then, the reversed reactions are caused by some change in the metabolic processes of the animals. Potassium cyanide depresses the metabolic processes by decreasing oxidation. Oxidation is decreased by

decreasing the ability of the tissues to take up oxygen. Chloretone is a soporific and has a depressing or inhibiting effect upon certain metabolic processes. Starvation decreases metabolism by removing the material to be oxidized. Such reagents and treatments are known to have specific effects on metabolism and they also cause reversals in phototaxis, therefore the responses are related to the metabolic rate of the animal.

Jennings ('04) says that physiological states are the most important determining factors in reaction and behavior. By physiological states he means the varying physiological conditions as distinguished from permanent anatomical conditions. Can we be sure that such physiological states do exist? If we subject animals to the same external conditions and give the same stimulus, and the animals react differently, then the difference must be due to variations in internal conditions; else the reactions would always be the same. A stimulus changes the physiological state of the animal as a whole, and this change in physiological state induces a certain type of reaction.

My thanks are due to Dr. V. E. Shelford under whom this work was done, and to Dr. W. C. Allee and Mr. M. M. Wells for valuable suggestions.

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A CASE OF PERSISTENT MELANISM.

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The occurrence of melanism is a phenomenon of wide distribution in nature, being recorded among animals belonging to a great many classes and orders. Our records show that while the occurrences are frequent and are found in species belonging to many of the larger zoölogical groups, yet the actual numbers of melanic individuals found among the individuals of any one species, in any one region is usually extremely small in comparison with the total number of normally colored individuals of the same species found in the same region. Because of this rarity of these black-colored individuals the appearance of melanic forms has been very generally regarded as being due to sporadic though at times oft-repeated, sporting. Such melanic forms do not usually persist racially. It was Darwin who years ago noted that sports of almost all kinds were ruthlessly eliminated in the struggle for existence; and sporting in the form of melanism apparently has offered no exception to this general rule.

Perhaps one of the best examples of the racial persistence of melanism is that of the melanic form of the moth, *Amphidasys betularia*, which existed as a rarity in England some years ago, but which now has replaced the typical form about some of the manufacturing districts. This persistence of melanism has been explained on account of the environment in these districts being changed by the smoke from factories which darkens the vegetation in general, by the killing of lichens and by the depositing of black soot, and in this manner gives an advantage to the melanic forms by making them less conspicuous than the normally colored individuals.

The Appearance of Melanic Rose Curculios.

During the summer of 1913, while in the Willamette Valley in western Oregon, I came across instances of melanism among

individuals of one of our common beetles that were quite striking. We have in the Willamette Valley, as in almost all other sections of the country, the well-known rose curculio, *Rhynchites bicolor* Fab., which feeds chiefly upon the buds and flowers of wild and of cultivated roses. It will feed, however, upon a few other plants, especially the buds of wild blackberries, which grow so abundantly along the streams in western Oregon.

This weevil is about one fourth of an inch long, and in all the sections of the country where it has been observed outside of the Willamette Valley is of a red color above; while the underparts of the body, and sometimes the head and beak, are black. The red in some instances extends forward so as to include the head and beak. When viewed feeding on the roses, the dark under surface of the body is largely concealed so that the weevil appears almost entirely red.

The red color of the dorsal surface is possibly protective to the species when it is feeding on the petals of wild or cultivated roses, as the color harmonizes with the red of many roses, and for this reason might make the individuals much less conspicuous objects to hungry birds and other enemies.

I found feeding along with the red individuals of *Rhynchites bicolor* Fab. individuals which were totally black. At first I suspected that these black individuals were of a different species. Upon looking the matter up I found that they had in a few instances been collected, and were called *Rhynchites æneus* Fab., a black species, which is common in the eastern part of the country.

During the summer of 1913 I demonstrated that the two forms would breed together in captivity. However, of the several larvæ that I obtained from the eggs deposited none reached maturity. Following these experiments I made a critical study of the characters of our two forms found in Oregon, and failed to observe any differences in structure whatever, hence considered the black ones only as melanic individuals of the common rose curculio. In order to get the opinion of a specialist in Coleoptera, I showed specimens of the two forms to Dr. Van Dyke, of the University of California. He stated that the two were the same, and that the black form found in Oregon was not the *Rhynchites*

æneus Boh. of the eastern states. I may here add that the real *Rhynchites æneus* Boh. is a very hirsute species; it has well-developed punctate striations on the elytra, and prominent marginal elytral grooves. Our black Oregon form has none of these characters pronounced,—the hairs are very small, the punctations in the elytral striæ are almost obsolete, and the marginal grooves of the elytra can hardly be noticed.

Mating and Other Habits.

During the spring and summer of 1914 I made many field notes on these two forms of *Rhynchites*. I found that the two were constantly associated. They fed together in the same way on the same rose bushes and even on the same buds. They were found to feed together on wild blackberries; they emerged from winter quarters at the same time, and, finally, they were repeatedly found to be interbreeding in nature.

Attempts at Rearing Hybrids.

Four of the individuals of the black form which were found mating in nature with red individuals were confined with their red mates in separate breeding cages. Here they continued to breed for over a month, after which they began to die. In the meantime the females had laid large numbers of eggs; but out of this large number, including several score, I am very sorry to state that I did not succeed in rearing a single individual to maturity. The whole trouble this year was that the buds of the roses soon died after they were punctured by the female, and fell to the ground, taking with them the developing larvæ. In these dead, shriveled rose-buds the larvæ invariably died. Even their transference to fresh rose-buds was of no use, for they would not stay in these buds when placed there artificially. None of these larvæ pupated.

It may be well to state that in all the literature which I have examined I have failed to find a single record of this weevil being reared from the egg to its adult state. Its life history has not been worked out by complete breeding experiments, although repeated field observations, together with fragmentary notes on the life history, have shown that it produces but a single brood a year and hibernates in the adult stage.

Mating Statistics.

Having been foiled in my attempts at breeding these beetles, I decided to attack the problem from a different standpoint, namely, to observe the mating habits in nature, to see if the black individuals mated as freely with the red ones as with those of their own color. During the months of May and June I made fourteen different observational trips to some patches of wild roses at the west end of the town of Corvallis, Oregon. I collected every pair that was observed mating, as well as those that were not observed mating; these were collected so as to obtain population statistics. The results obtained were as follows:

Total number of matings observed	53
Normal *Rhynchites bicolor* × normal *Rhynchites bicolor*	44
Melanic form × melanic form	0
Normal *R. bicolor* ♂ × melanic form ♀	3
Melanic ♂ × normal *R. bicolor* ♀	9

Thus we find that out of the total number of matings observed in nature, 83 per cent. were between normally colored individuals, while 16 per cent. were between melanic individuals and normal ones; no matings being observed between two melanic forms.

Although we can not prove anything in regard to the exact status of these two forms of *Rhynchites* from these observations alone, yet we may speculate, somewhat, in regard to a few points. They tend to indicate:

1. That the normal intergeneration of these two forms is followed by the segregation of the characters following the dominance in the first cross.

2. That the black form is recessive to the red.

3. That racial characters have become fixed without natural barriers, without isolation of any kind or without change of habit but purely through the segregation of the characters in the germ plasm.

Population Statistics.

The population statistics obtained for the same patches of wild roses that the mating statistics were obtained from, and at the same time are as follows:

Rhynchites bicolor Fab., normal	588
Black form	68
Total for both forms	656

Thus out of the total of 656 individuals counted, 68, or 11 per cent. were melanic. This percentage is so large that I think that no one would suspect the occurrence of melanism in this species to be due to the sudden sporting of a great many individuals, but rather to the persistence of melanism through segregate inheritance of the melanic types from a few or even a single sport progenitor. In other words either melanism or other characteristics associated with it in this instance have been advantageous to the black forms under the particular conditions of the Willamette Valley to such an extent that a black or melanic race has been evolved in direct competition with the normal type of the species from which it sprang, and has not been ruthlessly eliminated as a race as is usually the case with melanic forms.

The future of this black race of rose curculios will be interesting to watch. I hope to be able soon to breed these forms successfully, and also hope to be able to ascertain what the conditions are in the Willamette Valley which apparently make melanism advantageous to this curculio. A description of this melanic form is here given. Even in the Willamette Valley its distribution differs from that of the normal *R. bicolor*.

Rhynchites pullatus new variety, or species.

All the individuals as yet observed are black throughout. Body and appendages clothed with very fine short hairs which are not noticeable with the naked eye. Snout subequal to the tibia of the first pair of legs in length, bent almost straight downward in the male, but extending forward so as to be plainly visible from above in the case of the female. The antennæ arise from a position a little in front of the middle of the snout in the male and a little behind the middle of the snout in the female. Antennæ well clothed with hairs; three distal segments much broader than the others and forming a club. Dorsal surface of thorax punctate. Around the margins of each elytron is a distinct groove. The longitudinal punctate lines are rather indistinct, and may be wanting. Femora of anterior legs somewhat swollen. Length of body excluding snout, 5.5–7.0 mm.

STUDIES OF FERTILIZATION.

VII. Analysis of Variations in the Fertilizing Power of Sperm Suspensions of Arbacia.

FRANK R. LILLIE.

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I. Introduction.

In his epoch-making "Expériences pour servir a l'histoire de la génération des animaux et des plantes" published in 1785 the Abbé Spallanzani describes among his numerous experiments on fertilization and artificial parthenogenesis some determinations concerning the minimal quantity of sperm necessary to fertilize the eggs of the frog. He found that he could get perfect fertilization with seminal fluid diluted 2,720 times with water. At greater dilutions the percentage of fertilized eggs began to fall off, but some eggs fertilized up to a dilution of about 20,000 times. He calculated that the weight of the "spermatic particles" necessary to fertilize an egg was 1/2,994,687,500 of a grain, and that the volume of the egg in proportion to the volume of spermatic particles necessary to fertilize it is as 1,064,777,777: 1. In 1824 Prevost et Dumas confirmed these calculations.

So far as I know such experiments have not been repeated. The reason for this would appear to be that when it was once established that only a single spermatozoön unites with each ovum in fertilization all such quantitative studies of the fertilizing power of sperm dilutions appeared to have lost their point. So long as it was assumed (as was generally the case) that the fertilizing power of the spermatozoön is a function of its motility

alone, that is, of its capacity to "penetrate the ovum," there could be no object in quantitative studies. But as it came to be recognized that the fertilizing power of the spermatozoön is associated with some definite substance that it bears, possibly either a lysin (Loeb) or an activator (self), the problem assumes a different aspect; for it is obvious that if the sperm should lose such a substance in any way, its fertilizing power would be lost even though its motility should be preserved unimpaired. In such a case the relative fertilizing power of sperm suspensions could not be measured either in terms of concentration or of activity of the spermatozoa. Variations in the fertilizing power of suspensions of known concentrations might, therefore, be a measure of the loss of the postulated fertilizing substance. On reflection it is obvious that the spermatic substance in question must be loosely bound to the sperm, because it exerts its first effect, that of inducing cortical changes in the egg, before penetration, as I have shown for *Nereis*, and Loeb for certain hybrid combinations; at this time, therefore, the spermatozoön must set free its receptors[1] (activators).

Recently Glaser (1913 and 1915) has maintained that in *Arbacia* more than one spermatozoön is needed for fertilization of the egg, even though only one actually penetrates. The observations on which this conclusion rests are no doubt correct, *under the given conditions*, and I have made similar observations, as will appear in the course of the present paper. But it by no means follows from the observations that a single spermatozoön may not be adequate under other conditions (and this can be demonstrated). We cannot, however, deny *a priori* the possibility that for the initial phases of fertilization a number of spermatozoa may be of assistance though only one enters and is concerned in later phases. If the phenomena of fertilization are to receive a physiological, and ultimately a chemical, inter-

[1] In study VI. (1914), I propounded a theory of fertilization according to which the initiation of development of the egg is due to activation of an ovogenous substance, which I named fertilizin, contained in the cortex of the egg. In fertilization such activation is caused by a certain constituent of the sperm, which I called the sperm receptors; and the action of the fertilizin thus aroused must be on certain substances of the egg which I named in general egg-receptors. From a chemical point of view therefore we must have an interaction of three substances (or groups of substances), viz., sperm receptors, fertilizin, and egg receptors.

pretation, quantitative questions may be of serious significance.

It would seem to be a perfectly simple matter to determine the greatest dilution of sperm at which any fertilization takes place, and to express in the form of a curve, from percentages of eggs fertilized, the rate of loss of fertilizing power due to dilution. This was the very simple problem with which the present investigation began. However the results were in the highest degree contradictory; the same lot of sperm might vary in a period of half an hour from 1/1,024 to 1/9,000,000 (or less) of 1 per cent. dilution in its power to fertilize the same percentage of a single lot of eggs. The investigation, therefore, turned to the problem of such variations and their cause.

II. Experiments.

1. *Methods.*

Quantitative methods cannot possibly be as rigorous in a problem of this kind as in a purely chemical problem. In the first place we have to deal with variable reagents in the ova and sperm of *Arbacia;* and in the second place the initial measurements must be made rather hurriedly, so as to ensure freshness of the reagents, and under conditions that do not injure their vitality; the available quantities of material also limit the methods of measurement.

Sperm.—The standard for measurements of sperm dilutions is the "dry sperm"; *i. e.*, the thick creamy mass that exudes from ripe testes of *Arbacia.* If a ripe male be opened and inverted in a dry Syracuse watch crystal a certain amount entirely free from foreign admixture usually flows from the genital pores and collects in a mass in the crystal. While this may in certain cases be as much as 2 c.c., usually it is a much smaller quantity. It is quite impracticable to measure this by graduated pipettes; I have therefore used a drop of this dry sperm from bulb pipettes of fairly uniform openings as a standard, and, reckoning 30 such drops to the cubic centimeter, have made "1 per cent. sperm suspensions" by the addition of such a drop to 3.3 c.c. of sea water. This is the standard suspension from which most of the experiments proceed, and all sperm suspensions are expressed in fractions of such a 1 per cent. suspension. Given perfectly

dry sperm, the initial variation due to the method cannot be very great in relation to the tremendous range of variation in fertilizing capacity of sperm due to other causes. Indeed it is a vanishing quantity.

Eggs.—Egg concentration is a factor of relatively slight significance within the limits of the experiments, as will appear from the facts to be presented. Within a very wide range it does not affect the result measured in percentage of fertilized eggs. It is measured roughly by allowing washed eggs to settle for half an hour in a 100-c.c. graduated cylinder, and expressing the quantity there settled as a percentage of the entire fluid. There is of course for every concentration of sperm an egg concentration that is above the optimum for percentage of fertilization. But, as will be seen from the tables, such egg concentration lies beyond the concentration used in most experiments.

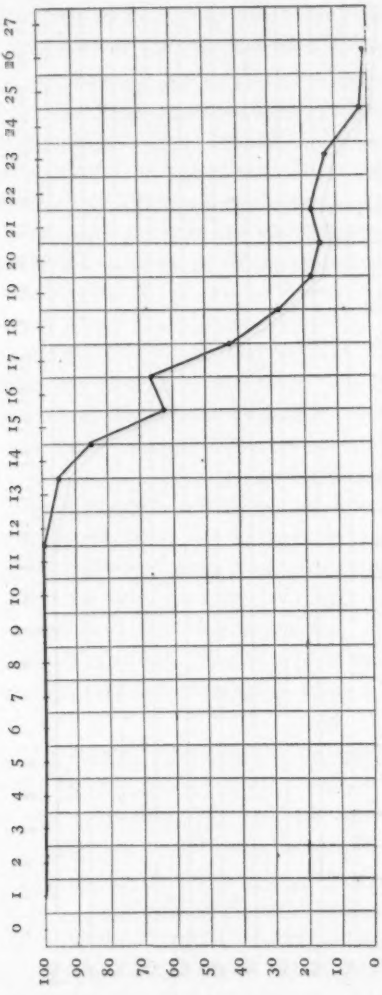
In most cases segmentation of the eggs was used as the criterion of fertilization, but membrane formation was also used in some cases, especially in high concentrations of sperm where many eggs failed to segment owing to polyspermy.

Formulation of Results.—The fertilizing power of sperm suspensions is expressed in curves whose ordinates are percentages of fertilization, and the abscissæ a geometrical series of dilutions of 1 per cent. sperm in powers of 2. This method was adopted for the abscissæ because of the method of successive half dilutions used in many experiments, and because the enormous range of fertilizing power made it impossible to compare results on one scale with an arithmetical progression. When it is realized that the fertilizing power may cease at 1/156 of 1 per cent., or extend to 1/90,000,000 the necessity of the geometrical series in the abscissæ will become apparent.

2. *The Optimum Curve of Dilutions.*

We may begin with the optimum curve of dilutions (Curve 1), because this answers most completely, and probably fully, to the current expectation that a single spermatozoön suffices for the fertilization of an egg. This curve is prepared from data of experiments calculated to bring eggs and sperm together in the freshest possible condition of the sperm. In general measured

quantities of washed eggs were put in measured amounts of sea-water, and measured quantities of definitely calibrated sperm suspensions added and stirred in as uniformly as possible.



CURVE 1.

A control of unfertilized eggs in sea-water was always kept to guard against chance fertilizations. To illustrate: the last four determinations of the curve were made as follows: In four

crystallization dishes were placed 1,000 c.c. sea-water (*A*), 3,000 c.c. sea-water (*B*), 1,000 c.c. sea-water (*C*), 3,000 c.c. sea-water (*D*). To each was added 2 c.c. of a washed egg-suspension (about 3 per cent. to 5 per cent.). The sperm was then prepared as follows: (1) one drop dry sperm to 3.3 c.c. sea-water at 9.43 A.M. = 1 per cent.; (2) 1 c.c. of sperm 1 to 99 c.c. sea-water 9.43.30 A.M. = 1/100 per cent.; (3) 1 c.c. sperm 1 to 999 c.c. sea-water 9.45.30 = 1/1000 per cent. To *A* was added 1 drop sperm 2(1/100 per cent.) at 9.43.45; to *B* one drop sperm 2(1/100 per cent.) 9.44; to *C* one drop sperm 3(1/1000 per cent.) 9.45.45; to *D* one drop sperm 3(1/1000 per cent.) 9.45.45. An assistant stirred in the sperm thoroughly as added. The sperm concentration in *A* was therefore $1/100 \times 1/30 \times 1/1000 = 1/3{,}000{,}000$ per cent.; in *B* it was 1/9,000,000 per cent.; in *C* 1/30,000,000 per cent.; in *D* 1/90,000,000 per cent. 1/3,000,000 per cent. falls between 21 and 22 on the scale, and the others as shown. The exact times of mixing the sperm are given because, as will appear beyond, time is an extremely important factor with reference to fertilizing power.

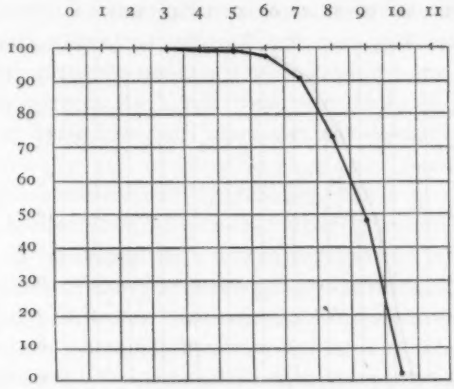
To appreciate the extent of this dilution it may be said that beyond a dilution of 1/10,000 per cent. (between 13 and 14 on the scale) one can rarely find a single spermatozoön in the jelly of the fertilized eggs. At about 1/2000 per cent. (11 on the scale) the sperm suspension does not even appear opalescent. We may therefore feel reasonably sure that beyond about 14 or 15 on the scale a single spermatozoön certainly suffices to completely fertilize an egg.

In further elucidation of the curve I may say that the critical (steep) part was covered by several determinations for each point. Thus there are five determinations averaged for the positions between 13 and 15. Seven between 15 and 18, five between 18 and 20, and six between 20 and 21. The determinations beyond 21 are single determinations. For the first part of the curve up to 13, there are numerous determinations. There are great variations in the single determinations compared with one another; these averages must therefore be regarded only as approximate values. With a sufficiently large number of determinations the irregularities between 15 and 17 and between 19

and 22 would no doubt disappear. But it is improbable that the general form of the curve would undergo any essential change even with a much more extensive series of determinations.

3. *Curves of Successive Half-dilutions.*

In contrast to these results, and for the purpose of defining the character of the main problem sharply, we may next consider the fertilizing power of a series of half dilutions of a 1 per cent. sperm suspension. The curves from these experiments furnish an almost incredible contrast to the one already given; as an example we may examine the following strikingly regular curve, Fig. 2. The first member of this series was a 1/8 per cent.



CURVE 2.

sperm suspension freshly prepared, thus falling in position 3 on the scale; 8 c.c. of this was taken (No. 1); to 4 c.c. of 1, 4 c.c. of sea-water was added (No. 2) = 1/16 per cent., ($1/2^4$); to 4 c.c. of 2, 4 c.c. of sea-water was added (No. 3) = 1/32 per cent., ($1/2^5$); this was continued eight places to $1/2^{10}$. Four drops of a 10 per cent. egg-suspension was then added to each, and the percentage of segmented eggs was counted three hours later. Plotted they give the above curve. In this case it will be seen that the fertilizing power almost ceases at $1/2^{10}$ = 1/1024 per cent. sperm suspension. The eggs and sperm were not at fault

because a parallel control series, in which the same quantities of the same lot of eggs were first placed in the same quantities of sea-water and sufficient of the original 1 per cent. sperm suspension added to make similar sperm dilutions, showed over 95 per cent. cleavage in each case, and actually 99 per cent. in No. 8 of the control where the sperm dilution was 1/1200 per cent. As a further control it may be added that eggs which fail to fertilize in such relatively concentrated sperm suspensions may all be fertilized by the subsequent addition of a trace of perfectly fresh sperm.

The type of experiment just cited was the first undertaken, and for a time it seemed to offer an almost insoluble problem, though the real explanation turned out to be extremely simple. I have twenty curves from similar experiments, fourteen of which run out absolutely from the third to the twelfth place on the scale (*i. e.*, from 1/8 per cent. to 1/4096 per cent.); in the remaining 6 (as in the curve just given) the dilutions were not carried far enough to reach the zero point, but they agree in principle with the others.

A number of control experiments demonstrated the relative lack of significance of the actual sperm concentrations. As one of these I may mention experiment C of August 3. In this case a series of sperm dilutions in powers of 4 was made from 1 per cent. The proportion of eggs fertilized ran off to 1 per cent. at $1/4^5$ ($1/2^{10}$) and to 0 at $1/4^6 = 1/4096$ per cent. But one drop of a 0.1 per cent. suspension of the original 1 per cent. sperm added to eggs in 200 c.c. sea-water fertilized 94 per cent. of them (control for sperm). Thus the control fertilized almost perfectly at 1/60,000 per cent. dilution, whereas the fifth member of the series of dilutions $1/2^{10}$ (1/1024 per cent. sperm) fertilized only 1 per cent. The actual concentration of the sperm is thus not the most significant thing.

This is also brought out strikingly in the following experiment (August 14). A series of half sperm dilutions was made as usual (Series *A*) 2 c.c. in each dish; to a second series (series *B*) of dishes was added 2 c.c. sea-water each and 4 drops of an egg-suspension. The numbers of series *B* were then inseminated by one drop each of sperm from the corresponding number of *A*, thus

diluting the sperm about 1/60. To each of the *A* series (except 1) four drops of the same egg-suspension was then added. The resulting percentages of fertilization are given in Table I.

TABLE I.

A.		*B*.	
1. (1%)	–	1. (1/60%)	– 99%
2. (1/2%)	– 96.5%	2. (1/120%)	– 99.5%
3. (1/4%)	– 99.5%	3. (1/240%)	– 98%
4. (1/8%)	– 99%	4. (1/480%)	– 60.5%
5. (1/16%)	– 98.5%	5. (1/960%)	– 51%
6. (1/32%)	– 96.5%	6. (1/1920%)	– 8.5%
7. (1/64%)	– 21%	7. (1/3840%)	– 1.5%
8. (1/128%)	– 6%	8. (1/7680%)	– 0
9. (1/256%)	– 3.5%	9. (1/15360%)	– 0

If we compare *A* and *B* in this table it will be seen that while it is true that *B* runs out earlier than *A*, nevertheless the fertilizations in the two series are not proportional to concentrations of sperm; for instance *A* 9 at 1/256 per cent. fertilizes 3.5 per cent. of the eggs, whereas *B* 3 at 1/240 per cent. fertilizes 98 per cent., *B* 4 at 1/480 per cent. fertilized 60.5 per cent., *B* 5 at 1/960 per cent. fertilizes 51 per cent. It is obvious that it is not concentration but *condition* of the sperm that is significant, which comes out with extreme emphasis in a control of this series. In this control, 1 drop of *A* (1 per cent. sperm) was added to 8 drops of the same egg suspension in 1,000 c.c. of sea-water 2 minutes after the other inseminations, thus making a 1/30,000 per cent. (1 × 1/1000 × 1/30) sperm suspension; every egg fertilized; the percentage of cleavage was 100 per cent.

The question then arises, what is this condition of the sperm which causes such loss of fertilizing power? We may note the following points: (1) To bring out the lack of significance of the absolute concentration of the sperm, in several of the experiments with successive half dilutions, counts were made of the numbers of spermatozoa seen in the egg-jelly of members of the series with no fertilizations: Thus on July 16 a series of half dilutions ran out to 0 in the seventh crystal (1/128 per cent. sperm): in ten eggs selected at random from this crystal, an average of 9 spermatozoa was counted in the jelly and in contact with the membrane of these eggs; but, as the upper and lower surfaces

could not be examined, the whole number must have been at least double; in No. 8 of the series, an average of five spermatozoa was counted with each egg; in No. 9 an average of 1.2; No. 10, 1.4; No. 11, 0.9. Similar counts were made in other cases. But in fertilizations under optimum conditions all of the eggs may fertilize in dilutions of sperm so great that it is almost impossible to find spermatozoa in the jelly of the eggs. (2) The spermatozoa are active and the eggs readily fertilizable in such a series as the above. Repeated observations were made on this point; which would be tedious to relate in detail.

It may be noted that in the fertilization under optimum conditions the eggs were first placed in sea-water, and given quantities of sperm then added; whereas in the experiments with successive dilutions eggs were added to sperm suspensions already made up. This suggested that the order of adding eggs and sperm might be of significance in some way. However, this does not appear to be the case.

The possibility remained that the repeated handling of the sperm in successive dilutions decreased their motility. Microscopical examination did not confirm this idea; and subsequent experiments disproved it, as the fundamental factor at least.

Thus it would appear that the only real difference between the optimum and minimum conditions of the fertilizing power of sperm dilutions is a time factor; under what I have called the optimum conditions the final dilution is made from a relatively concentrated sperm suspension in the presence of eggs; but under the conditions of successive dilutions time elaspses before the eggs are added.

Thus in Curve 2 the preparation of the series of sperm dilutions from the original 1 per cent. suspension occupied 22 minutes before the eggs were added. In Curve 1, on the other hand, less than a minute elasped from the time of preparing the 1/100 per cent. and 1/1000 per cent. sperm suspensions used in the last four determinations to the time of their use in inseminating (see p. 234); and the final dilution was made in the presence of the eggs.

The time factor is the real explanation as will be shown immediately. But at first sight it did not seem a very probable

explanation for two reasons: in the first place the time from preparation of the original 1 per cent. sperm suspension to that of addition of eggs is usually less than twenty-five minutes, which is usually considered too short a time for injury to sperm; and in the second place, after the addition of eggs to the sperm-dilution series, in several control experiments the original 1 per cent. sperm suspension was shown to be capable of fertilizing at 1/30,000 of 1 per cent. ($1/2^{15}$ ca.) by addition to eggs in sea-water. *If the sperm suspensions lose their fertilizing power with time, it must be that the significance of time in this respect varies inversely to concentration.* As soon as such a proposition is formulated it is easily tested experimentally, and this was done in a thorough fashion.

4. *Time as a Factor in the Fertilizing Power of Sperm Suspensions of Different Concentrations.*

The experiments under this head were performed in three ways: *A*. A considerable quantity of the sperm suspension to be tested was made up, and divided in several equal parts in a series of bowls; measured equal quantites of the same egg-suspension were then added to members of the series at definite time intervals. This method was followed for sperm dilutions from 1/300 per cent. (between 8 and 9 in the scale) down. *B*. Measured amounts of the more concentrated sperm suspensions were added at time intervals to measured quantities of eggs in equal amounts of sea-water. *C*. Finally, to control the data in section 3, a series of sperm suspensions, made by successive half dilutions as in section 3, was divided in two equal series, and eggs were added at once to the one series, and after a time interval to the second.

A. The following table gives the data under method *A*. The figures at the head of each vertical column give the sperm dilution in fractions of the 1 per cent. sperm suspension; below is given the place of such a sperm suspension in the scale of powers of 2. The figures in the columns give the percentages of fertilizations for inseminations made at the time (age of the suspensions in minutes) indicated at the left. To illustrate the method of experimentation for one column which will serve for all the rest,

TABLE II.

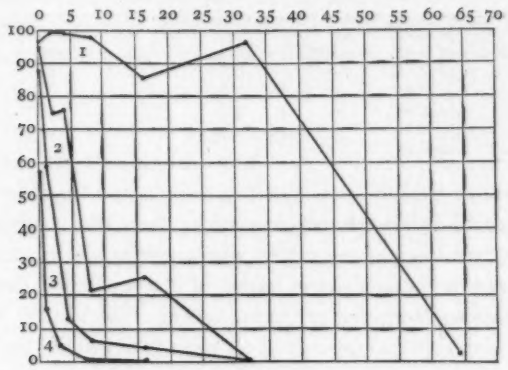
SHOWING RATE OF LOSS OF FERTILIZING POWER OF SPERM SUSPENSIONS.

	1/300	1/600	1/1200	1/2400	1/3000	1/3600	1/6000	1/12000	1/18000	1/24000	1/30000	1/60000	1/120000	1/240000
0	96	100	99.5	95	95.5	97	91	97	....	97.5	88	91.5	58	40
1	....	....	99.5	49.5	.....	52	.....	....	....	.....	....	42	16	6
2	99.5	98.5	.....	....	75	....	80	92.5	98	99.5	59.5	....	.....	....
3	....	....	100	91.5	.....	88.5	.....	.....	....	.....	....	....	4	16
4	99.5	95.5	.....	....	76	....	34.5	93.66	85.5	49.5	13	15.5	.....	....
6	....	....	93.5	52	.....	80.5	.....	.....	....	.....	....	....	0.5	1
8	98.5	65.5	.....	....	21	....	28.5	54.5	73.5	67	7	17	.....	....
16	85.5	?	99.5	45	26	16.5	.....	.....	14	36	4.5	1	0	0
32	96.5	67.5	30.5	1.5	0	4.5	9	1	0	4	0	0.5	0	0
64	2.5	19	0	0.5	0.5	3.5	2	0	0	4	0	0	0	0
120	8.5	3	.....	....	.....	....	.....	.....	....	.....	....	....	.....	....
	8–9	9–10	10+	11+	11–12	12−	12–13	13–14	14+	14–15	15+	16−	16–17	18−

we shall give the experimental data for 1/30,000 per cent.: August 18, 1914. The eggs of two females were taken at 9.50 A.M. and washed at 10.04, 10.06 and 10.23 (150 c.c. of sea-water being used in each washing). A series of 7 Syracuse crystals was then laid out with 10 c.c. of sea-water in each. To 1 was then added 5 drops of the egg-suspension. A single drop of fresh dry sperm was then added to 333.3 c.c. sea-water at 10.37 making a 1/100 per cent. sperm suspension, and 1 drop of this was added to crystal 1 at 10.37.30 and stirred in by an assistant making a 1/30,000 per cent. (1/100 × 1/10 × 1/30) sperm suspension in presence of eggs. One drop of the 1/100 per cent. sperm was also added to crystals 2–7, which contained no eggs, at 10.38, making 1/30,000 per cent. sperm suspension in each. To No. 2, 5 drops of the same egg-suspension was added at 10.40, to No. 3 at 10.42, to No. 4 at 10.46, to No. 5 at 10.54, to No. 6 at 11.10, to No. 7 at 11.42. At 2.30 P.M. my assistant, Mr. Cohn, then estimated the percentages of segmented eggs in each crystal, by first thoroughly mixing the eggs, then assembling them, taking a sample, and making two counts of 100 each, which were averaged.

The table shows (1) that the effect of time up to 64 minutes is to diminish the fertilizing power of the suspensions at every dilution represented. (2) That the rate of loss of fertilizing

power increases with dilution, *i. e.*, the effect of time varies inversely to concentration of sperm. This is brought out very clearly by the following curves (Fig. 3) of loss of fertilizing



CURVE 3.

power of sperm suspensions at different concentrations. The abscissæ represent age of sperm suspensions in minutes; the ordinates represent fertilizing power as expressed in percentages of segmenting eggs. Each curve stands for a given sperm dilution. Curve 1 represents loss of fertilizing power of a 1/300 per cent. sperm suspension, curve 2 of a 1/3000 per cent., curve 3 of a 1/30,000 per cent. and curve 4 of a 1/120,000 per cent. sperm suspension.

B. On August 6, I prepared a series of seven sperm dilutions in powers of 4 from 1 per cent. to 1/4096 per cent. Each of these was then used to fertilize a measured quantity of egg-suspension at the intervals given in Table III.

For the fertilizations 10 c.c. sea-water was measured out in advance in Syracuse crystals and 5 drops of a 5 per cent. egg suspension added to each. For each fertilization 1 drop of sperm was added and stirred in. It will be observed that 1 per cent. sperm lost none of its fertilizing power so far as this test went; 1/4 per cent. fell off from 96.5 per cent. to 16.3 per cent.; 1/16 per cent. from 46.5 per cent. to 0; 1/64 per cent. from 0.5 per cent. to 0 in the second place; whereas the greatest dilutions did

TABLE III.

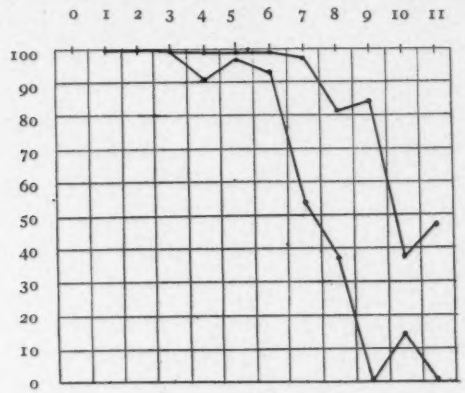
Sperm Dilution.	Made at	Fertilizations.				
		A.	*B.*	*C.*	*D.*	*E.*
		2.50 P.M.	3.01 P.M.	3.16 P.M.	3.35 P.M.	4.03 P.M.
1. 1%.....	2.20 P.M.	99 %	100%	100 %	99.5%	99.5%
2. 1/4%.....	2.28 P.M.	96.5%	68%	61.5%	18.5%	16.3%
3. 1/16%....	2.30 P.M.	46.5%	18%	8.5%	4 %	
4. 1/64%....	2.32 P.M.	0.5%	0%	0%	0 %	
5. 1/256%...	2.34 P.M.	0	0%	0%		
6. 1/1024%..	2.36 P.M.	0	0%			
7. 1/4096%..	2.39 P.M.					

not fertilize at all. It should of course be noted that the sperm suspensions used were diluted 300 times in the actual insemination (10 c.c. = 300 drops of sea-water, and one drop sperm added).

Thus time is an important factor in the fertilizing power of sperm dilutions from 1/4 per cent. down. The matter cannot be stated with great accuracy, but we can say in general (referring to Table II.) that sperm suspensions from 1/300 per cent. to 1/1200 per cent. lose their fertilizing power nearly completely in 64 minutes; from 1/2400 to 1/24,000 in 32 minutes; from 1/30,000 to 1/60,000 in 16 minutes; from 1/120,000 to 1/240,000 in 6 minutes. Table III. shows loss of fertilizing power of higher concentrations by a different method.

C. We are now in a position to understand the principal reason why the curves of successive half or quarter dilutions of a 1 per cent. sperm suspension run off so rapidly. The reason is that the preparation of the series requires time, 10 to 20 or more minutes. In the early experiments of this kind the significance of brief periods of time was not recognized, and so no time records were kept; but I have 13 curves with accurate time records. Of these I reproduce only two (Fig. 4). For this experiment (Sept. 5), (1) 6.6 c.c. of 1 per cent. sperm was prepared, 9.30 A.M. (2) 4 c.c. of 1 was transferred to a Syracuse watch crystal and 4 c.c. of sea-water added (= ½ per cent.). (3) 4 c.c. of 2 was transferred to crystal 3 and 4 c.c. sea-water added (= ¼ per cent.) and this was continued to 12 numbers (finished at 9.39.30 A.M.). The suspensions 2–12 was then divided in two equal amounts of 2 c.c. each, making series *A* and *B*. To each crystal of series *A* 2 drops of a 1 per cent. egg-suspension were

added at 9.47, that is, 17 minutes after the 1 per cent. sperm was prepared. Twenty-eight minutes later (10.15 A.M.) two drops of the same egg suspension were added to series *B*. The only difference between series *A* and *B* is the time factor. The percentages of cleavages were counted for both series, and the plotted results given the curves. Considerable loss of fertilizing power



CURVE 4.

has occurred in series *B* as compared with series *A*. Now, if we compare these time intervals with those given in Table II. we see that, in the curve of series *B* the last number, which is a $1/2^{11}$ or 1/2048 per cent. sperm suspension, loses its fertilizing power completely in 36 minutes (*i. e.*, from 9.39.30 to 10.15.30) which corresponds very well with the rate of loss of fertilizing power in a 1/2400 per cent. sperm suspension. This agreement is rather closer than usual; in some cases the series of ½ dilutions ran out at higher concentrations in about the same time; but in no case, I think, did they require more time. This suggests some possible stimulating effect of the successive changes which causes the spermatozoa to lose their fertilizing power more rapidly than under the time factor alone.

Gemmill (1900) observed that the duration of vitality of spermatozoa of sea-urchins and limpets tested by their movements or by the fertilizing capacity, varies greatly "according to the amount of sperm used in proportion to the volume of sea-

water in which it was shed." "When a small quantity of sperm was mixed with a large quantity of sea-water, the duration of vitality of the spermatozoa is short, but when the converse proportions are used, it is greatly lengthened." "By taking sperm from a sea-urchin and mixing it in different vessels with different quantities of sea-water, one obtains sets of spermatozoa, which will retain their vitality for a rising series of terms, *e. g.*, 8, 12, 16, 24, 48 and 72 hours. For the longest term, the proportion of spermatic fluid to sea-water should be not less than 1 to 10."

Gemmill was thus dealing with the same phenomenon with which we are concerned. He gives, however, no exact quantitative data and relatively few experiments were performed. He attributes the results to (1) greater activity of the spermatozoa, and consequent earlier exhaustion in the more dilute suspensions and (2) to dilution of the "spermatic fluid" by which he supposes the spermatozoa to be nourished.

5. *Other Factors in the Fertilizing Power of Sperm Suspensions.*

In the large number of experiments carried out to test the fertilizing power of sperm suspensions the general form of the curves is remarkably constant. Some, however, are quite irregular, and it was never possible to get *exactly* the same curve in the repetition of any experiment. A few of the irregularities may conceivably be due to error, as for instance the accidental presence of some toxic substance in one of the dishes of a series, though painstaking care was used to avoid such sources of error. The failure to obtain exactly the same curve in different experiments is no doubt also due in part to the natural variability of different lots of eggs and sperm.

In an attempt to discover the sources of variation and error, the effect of egg concentration, *i. e.*, the absolute quantity of eggs in a given bulk of a sperm suspension of given strength was tested. On the whole the effect of egg-concentration was found to be relatively small within so wide a range that it cannot be regarded as a large factor in the variability of the curves; because the egg-concentration of the curves was always below the point where it was demonstrably a limiting factor. Tests were made of sperm suspensions ranging from 1/62.5 per cent. to 1/8,000

per cent. But it was only from about 1/500 per cent. down that any considerable effect was observed within the range of egg concentration employed.

The method of the experiments tabulated (Table IV.) may be given for 1/500 per cent. sperm as it was the same for the others August 31: A quantity of 1/500 per cent. sperm suspension was freshly prepared 10.54.30 A.M., 2 c.c. of this was then placed in each of seven crystals (1–7). From 10.56.30 to 10.59 A.M. eggs were added as follows: to 1, one drop of a 1.75 per cent. egg-suspension, to 2 two drops, to 3 four drops, to 4 eight drops, to 5 sixteen drops, to 6 one c.c., to 7 two c.c. The numbers in the table give the percentages of segmented eggs. The tests with 1/1,000 per cent. and 1/2,000 per cent. sperm were made with the same egg suspension. For the tests with 1/4,000 per cent. and 1/8,000 per cent. sperm a 3.3 per cent. egg-suspension was used. Thus for each series the egg-concentration is approximately doubled in successive numbers of the series (in No. 7 = 64 times No. 1).

TABLE IV.

EFFECT OF EGG-CONCENTRATION ON THE FERTILIZING POWER OF SPERM SUSPENSIONS.

Sperm Suspensions.	Egg-suspensions.						
	1.	2.	3.	4.	5.	6.	7.
1/500%	100	99	99.5	97	93.5	82.5	56
1/1000%	97.5	94.5	93	76.5	48?	76.5	33.5
1/2000%	96.5	83.5	75	72.5	42.5	36	32
1/4000%	79.5	66.5	42.5	47.5	12.5	16	4.5
1/8000%	46.5	52	80?	30.66	27	15.5	7.5

The percentages of fertilization fall off in each of these sperm suspensions with increase of egg-concentration, and the amount of falling off increases in general with the dilution of the sperm. There was certainly no numerical deficiency of spermatozoa in the highest egg-concentrations; the reason for the falling off therefore appears rather obscure, and as it is not involved in the present problem, I shall not discuss it here. But as the egg-concentration employed in any of the preceding experiments did not exceed that of column 3, and the same egg-concentration

was always employed throughout any experiment, it is obvious that the effect to be attributed to the egg-concentration employed in the preceding experiments is very small.

III. Discussion.

Within a wide limit of egg-concentration the important factors in fertilizing power of sperm suspensions are: (1) concentration, (2) time. A third factor, which is not of equal significance to the other two, is the given variability of the reproductive elements. Such variability attaches of course both to ova and spermatozoa; in general it will affect only absolute values for given combinations, and not at all the relative values found in any single experiment. Moreover, as it is a chance factor, it will tend to be eliminated in a series of determinations. Fortunately both eggs and spermatozoa of *Arbacia* are relatively very constant materials if care be taken to wash the eggs thoroughly, and if the factors of concentration and time are fairly constant for the sperm. For the eggs these two latter factors are of such slight importance within the given limits as to be practically negligible. The significance of the concentration factor for the fertilizing power of sperm is of course obvious without discussion. We therefore turn to the time factor.

The most significant aspects of the time factor are, first, the unexpectedly rapid rate of loss of fertilizing power of sperm suspensions, and second the increase of rate of loss with dilution. There are but two ways of explaining these facts: either (1) the motility of the spermatozoa is quickly reduced in sperm suspensions to such an extent that they cannot bore into the egg or (2) the spermatozoa lose some substance essential for the fertilization reaction.

The following are the objections to the first alternative: (*a*) Microscopical examination lends it no support; I have repeatedly observed, that fertilizing power of sperm cannot be expressed either in terms of motility, or of success in penetrating the jelly of the egg and coming in contact with the membrane. In the experiments on successive half dilutions (p. 238) I kept records, in several series, of the numbers of spermatozoa in the jelly of unfertilized eggs, and found in some cases an average of 9

spermatozoa visible in the jelly, or on the membrane of certain lots of eggs none of which had fertilized; this could not be more than half of the spermatozoa in association with such eggs; and other observations made immediately after insemination demonstrated the high degree of motility of spermatozoa of entirely barren sperm suspensions.[1]

These observations contrast in the most striking manner with the fact that not a single spermatozoön can be seen in the jelly of eggs fertilized with highly dilute fresh sperm suspensions, where, nevertheless, nearly every egg may be fertilized.

(*b*) Penetration of the egg is not solely a function of motility of the spermatozoön. Penetration follows, as a matter of fact, after the fertilization reaction has begun, and it is due to the inception of such reaction, not the reverse as is commonly assumed.[2] In *Nereis*, as I have previously described, penetration does not take place until 45 to 50 minutes after insemination and the initiation of the fertilization reaction. The facts described in this paper show that in *Arbacia* no penetration takes place unless the sperm has started the fertilization reaction; if this does not take place, the spermatozoön remains external, however active it may be. And if it does occur the initiating spermatozoön is speedily engulfed by the egg.

(*c*) It is not easily understood on this theory why dilute sperm suspensions should lose their fertilizing power more rapidly than

[1] Glaser's experiments (1915) would bear the interpretation that, in those cases of normal insemination described by him in which fertilization does not occur except in the presence of several spermatozoa for each egg, the time factor which I have just described was operative. In other words that the majority of the spermatozoa in question had lost their receptors. But in the absence of exact data as to age and concentration of the sperm suspension, it cannot be asserted that this is the correct interpretation although I obtain exactly the same results in my time series (p. 238). My dilution experiments prove beyond a doubt that a single spermatozoon suffices for the whole process of fertilization under optimum conditions (defined on p. 233). Glaser's experiments, however, raise the question whether the efficacy of heavy insemination in the case of a stale sperm suspension is due to mass action, or to the survival of a small percentage of effective spermatozoa? So far as I can see this question can not be answered on the basis of our present information.

[2] Spermatozoa may penetrate into unripe ovocytes in some cases, as has been noted by several observers; in such a case there is no fertilization reaction. In the present experiments the unfertilized eggs were not penetrated by the spermatozoa.

more concentrated suspensions;[1] the relative freedom from CO_2 and other sperm excreta should favor a longer continuation of their motility in the dilute suspensions rather than the reverse.

(*d*) Moreover, in general the results of recent fertilization studies such as the antagonistic action of sperm suspensions of different phyla, inhibition of fertilization in the presence of blood of the species, or in the absence of certain ions (Loeb, '14), or again the sterility in certain self-fertilizations, and finally the inability of spermatozoa to penetrate fertilized eggs, unite in demonstrating the relative lack of significance of motility as such.

We come therefore to the conclusion that the individual spermatozoa in suspension tend to lose their fertilizing material, so that an increasing proportion of these spermatozoa become absolutely ineffective whatever their motility. This conclusion is in agreement with all the data of the foregoing experiments, and seems to be the only one competent to explain the results.

The following questions arise: (1) Whether the loss of this substance by the sperm is a mere process of diffusion or an active secretion? (2) Can the substance be recovered from the fluid of the suspension, or can its presence in the fluid be demonstrated in any way?

As regards the first question: In the case of the ova we know that the external jelly-covering is loaded with sperm-agglutinating substance which diffuses into the sea-water continuously. It is theoretically possible, at least, to apply a similar conception to the spermatozoön, although no such covering is demonstrable. The more rapid loss of fertilizing power in the greater dilutions would be consistent with this interpretation. From this point of view we would have to regard the sperm head as covered superficially with a layer of fertilizing material, like the phosphorus on a match. Such a conception is by no means impossible. On the other hand the fact that dilutions reached by a series of successive half-dilutions from 1 per cent. lose their

[1] Gemmill (1900) observed the same phenomenon and concluded that the more rapid exhaustion of spermatozoa in dilute suspensions is due to dilution of a hypothetical nutritive medium which keeps the spermatozoa of concentrated suspensions in a vigorous condition. This explanation comes back to the principle of loss of motility, so far as it relates to fertilizing power.

fertilizing power more rapidly than the same dilutions made in one stroke, indicates that successive stimulation hastens the loss, which therefore appears more in the nature of a secretion or a discharge than mere diffusion. The source of the substance must ultimately be the sperm cell itself, and it is quite possible that, as in the case of the egg, there is both a superficial layer and an internal supply.

It must be admitted that the data are inadequate to answer this problem. The statement of the problem can therefore serve only to bring out the resemblance between the spermatozoön and the ovum in respect to the existence of a fertilizing substance in each, the fertilizin in the case of the ovum and the sperm receptors in the case of the spermatozoön, and also the possible resemblance in respect to the disposition of the substances in each. It certainly is an interesting parallelism that both cells contain a substance necessary to fertilization, which may be lost in the sea-water.

The most interesting and crucial question of course concerns the possibility of detecting this lost substance in the fluids of the suspensions. If such a substance actually occurs in the fluid it should have the property of fertilizing ova; unless it can be detected by this property, we have no other indicator for it. So far I have not been able to make even a beginning on this problem. As is well known a number of experimenters have attempted without success to derive a fertilizing medium from spermatozoa. It has been suggested by Loeb that the reason for the failure to secure an extract of spermatozoa that will fertilize is that the motile power of the spermatozoön is needed to carry the effective substance into the egg. But it may equally well be that the methods hitherto employed have been too brutal; the substance may well be too labile to withstand extraction by ether, etc.

My results strongly suggest, if they do not prove, that such a substance must be present in the fluid of sperm suspensions of *Arbacia*, and they therefore suggest other methods for securing it for testing. We must bear in mind that it can form only an extremely small proportion of the entire spermatozoön, as proved by morphological considerations alone, and that it must

be superficial in position and easily detached as proved by its effectiveness before the spermatozoön penetrates. Extracts of the entire spermatozoön must contain numerous other substances which may neutralize its effectiveness.

The difficulty of the investigation as shown by my experiments is that it is liberated only very slowly in concentrated suspensions and that its amount in dilute suspensions would presumably be too slight to be effective. Some means can probably be devised for liberating it in concentrated sperm suspensions and freeing it of the spermatozoa for testing.

Finally I may point out that the conclusion that spermatozoa lose a substance necessary for the exercise of their fertilizing power is consistent with my own point of view of the mechanism of fertilization as well as with Loeb's. From my point of view the spermatozoön loses its receptors, viz., the substance that activates the fertilizin of the egg; from Loeb's point of view the spermatozoön loses its lysin, the substance that corrodes (cytolyzes) the egg.

My previous experiments had shown that eggs produce a certain substance in sea-water (fertilizin) which is necessary for their fertilization; fertilized eggs no longer produce this substance and are incapable of fertilization. Both eggs and spermatozoa therefore contain substances, more or less liable to loss, which are necessary for fertilization. The mechanism of fertilization cannot possibly, therefore, be regarded in the simple manner postulated by Loeb's theory. The existence of parthenogenesis demonstrates the efficacy under given conditions of the egg-substance alone; we must therefore regard the spermatic substance essentially as an activator of the fertilizin of the egg.

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